University of Alberta

Antigen Delivery to Dendritic Cells Using Biodegradable Poly(D,L-lactic-co-glycolic acid) Nanoparticles

by

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Dedications

This manuscript is dedicated to
My dear wife, Vijaya whose support and encouragement has been an invaluable resource for me.

My dear parents who instilled inspiration and confidence to me and encouraged me throughout the process.

Abstract

The focus of this dissertation is the evaluation of poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles as an efficient delivery system to target antigens to dendritic cells (DCs) for generation of cellular immune responses. PLGA nanoparticle formulations of MUC1 lipopeptide (BLP25) with or without immunomodulator, monophosphoryl lipid A (MPLA) were characterized with respect to size, BLP25 content, and antigen release. Immune response studies in human MUC1 transgenic mice *in vivo* showed that delivery of BLP25 and MPLA in nanoparticles elicited a strong T cell proliferative response with the secretion of IFN-γ by the activated T cells.

The ability of murine bone marrow derived DCs to internalize PLGA nanoparticles was next evaluated. DC primary cultures generated *in vitro* efficiently internalized PLGA nanoparticles through phagocytosis and this ability was influenced by the dose of the nanoparticles available in culture medium and the incubation time. Greater than 1000 fold uptake of the antigen by DCs was observed when the antigen was encapsulated in PLGA nanoparticles as compared to free form of antigen delivery.

Maturation of DCs following treatment with MPLA containing PLGA nanoparticles was next evaluated. MPLA nanoparticle treatment upregulated the expression of surface maturation molecules on DCs and enhanced their ability to stimulate allogeneic T cells. Furthermore, MPLA nanoparticle treated DCs secreted pro-inflammatory cytokines and chemokines.

The ability of DCs pulsed with ovalbumin (OVA) and MPLA containing PLGA nanoparticles to stimulate OVA specific T cells *in vitro* and *in vivo* was investigated. The results demonstrated that a strong antigen specific T cell proliferative response both *in*

vitro and *in vivo* could be achieved when OVA and MPLA were delivered in PLGA nanoparticles to DCs. Furthermore, DCs treated with BLP25 and MPLA containing PLGA nanoparticles stimulated the proliferation of T cells derived from human MUC1 transgenic mice.

This research demonstrates the capacity of PLGA nanoparticles to serve as delivery vehicles to efficiently target antigens and immunomodulators into DCs for induction of cellular immune responses and reveals the potential of DC based particulate vaccines in cancer immunotherapy.

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LIST OF ABBREVIATIONS

Ab	antibody
ABTS	2,2-azino-di(3-ethyl-benzthiazoline sulfonate)
ACN	acetonitrile
Ag	antigen
APC	antigen presenting cells
AUC	area under the curve
BCA	bicinchoninic acid
BLP25	MUC1 lipopeptide
BMDC	bone marrow derived dendritic cells
BSA	bovine serum albumin
C	celsius
cRPMI	complete RPMI
CCL	chemokine ligand
CCR	chemokine receptor
CD	cluster of differentiation
CD40L	CD40 ligand
CEA	carcinoembryonic antigen
CFA	complete freunds adjuvant
CFC	colony forming cells
CHL	chloroform
CLIP	class II associated invariant chain peptide
CLP	common lymphoid progenitor

CLSM	confocal laser scanning microscopy
CMI	cell mediated immunity
CMP	common myeloid progenitor
Con A	concanavalin A
Conc	concentration
CpG	cytosine-phosphate-guanine
CPM	counts per minute
Cs	Cesium
CTL	cytotoxic T lymphocyte
Cy5	Cyanin 5
DABCO	1.4-diazobicyclo-(2.2.2)-octane
DC	dendritic cells
DNA	deoxyribonucleic acid
DTH	delayed type hypersensitivity
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immunospot
ER	endoplasmic reticulum
FACS	fluorescent activate cell sorting
FCS	fetal calf serum
FDA	food & drug administration
FITC	fluorescene isothiocyanate
Flt3	fetal liver tyrosine kinase 3

FSC	forward scatter
g	gravitation force
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
HBcAg	hepatitis B core antigen
HBSS	hanks buffered salt solution
HCl	hydrochloric acid
Hep	hepatitis
HI	humoral immunity
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HPLC	high performance liquid chromatography
HSC	hematopoietic stem cells
HSP	heat shock proteins
HSLAS	health sciences laboratory animal facilities
I	iodine
ICAM	intercellular adhesion molecule
IFN-γ	interferon gamma
Ig	immunoglobulin
Ii	invariant chain
IL	interleukin
IP	interferon inducible protein
IRAK	interleukin receptor associated kinase

ISCOMS	immunostimulatory complexes
i.d.	intradermal
i.l.	intralymphatic
i.n.	intranodal
i.p.	intraperitoneal
i.v.	intravenous
K Da	kilodaltons
KLH	keyhole limpet hemocyanin
L	ligand
LAL	limulus amebocyte lysate
LAMP	lysozyme associated membrane protein
LC	langerhans cells
LPS	lipopolysaccharide
µl	microlitre
µg	microgram
mAb	monoclonal antibody
M-CSF	macrophage colony stimulating factor
MDC	myeloid dendritic cells
MFI	mean fluorescence intensity
mg	milligram
MHC	major histocompatibility complex
min	minutes
MIP	macrophage inflammatory protein

ml	milliliter
MLR	mixed lymphocyte reaction
MPLA	monophosphoryl lipid A
MR	mannose receptors
MUC1	mucin-1
mw	molecular weight
NaOH	sodium hydroxide
NEG	negative
NF-κβ	nuclear factor kappa beta
ng	nanogram
NK	natural killer cells
nm	nanometer
Np	nanoparticles
OD	optical density
ODN	oligodeoxynucleotide
OVA	ovalbumin
o/w	oil-in-water
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDCs	plasmacytoid dendritic cells
PE	phycoerythrin
pg	picogram

PG	prostaglandin
PI	propidium iodide
PLGA	Poly(D,L-lactic-co-glycolic acid)
pMHC	peptide-MHC
POE	poly(ortho esters)
POS	positive
PV	parvovirus
PVA	polyvinyl alcohol
RP-HPLC	reverse phase HPLC
RANTES	regulated on activation normal T cell expressed and secreted
rm	recombinant murine
RNA	ribonucleic acid
rpm	rotations per minute
RPMI	roswell park memorial institute
RT-PCR	reverse transcriptase-polymerase chain reaction
s	seconds
s.c.	subcutaneous
SDF	stromal derived factor
SDS	sodium dodecyl sulfate
SEM	scanning electron microscopy
SOCS	suppressor of cytokine signaling
Sol	soluble
SI	stimulation index

SLC	secondary lymphoid tissue chemokine
SSC	side scatter
TAA	tumor associated antigen
TAP	transporter of antigen presentation
TARC	thymus and activation related chemokine
TBAP	tetrabutylammonium dihydrogenphosphate
TCR	T cell receptor
Tg	transgenic
TGF-β	transformation growth factor beta
Th	T helper
THF	tetrahydrofuran
TIL	tumor infiltrating lymphocytes
TIR	TLR-IL-1 receptor
TIRAP	TIR domain containing adaptor protein
TLR	toll-like receptors
TMB	3,3',5,5' tetramethylbenzidine
TMR	tetramethylrhodamine
TNF-α	tumor necrosis factor alpha
TPBS	0.05% (v/v) tween 20/PBS
TRAM	TRIF related adaptor molecule
TRAF	TNF-receptor associated factors
T reg	regulatory T cells
TRIF	TIR domain containing adapter inducing IFN-β

TRIM	tripartite motifs
UV	ultraviolet
VEGF	vascular endothelial growth factor
VLP	viral like particles
w/o/w	water-in-oil-in-water
WT	wild-type

AMINO ACIDS

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
N	asparagine
P	proline
Q	glutamine
R	arganine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

Chapter One

Introduction

1.1 Introduction

The concept of modulating the immune responses by targeted antigen delivery has gained tremendous importance since the 1990's. Since then pharmaceutical delivery systems made of chemically defined materials such as biodegradable polymers have been developed and investigated for achieving this objective.

One of the cell types that plays a crucial link between the innate and adaptive immune responses and is of major interest in cancer immunotherapy is dendritic cells (DCs). DCs are professional antigen presenting cells (APCs) involved in the generation and maintenance of primary immune responses (1). DCs are capable of activating a wide variety of immune effector cells including T cells, B cells, natural killer (NK) cells, and NKT cells. Depending on the nature of the antigen and the microenvironment during antigen capture and presentation, DCs dictate the outcome of the immune response, which can be either immune activation or tolerance.

Given the pivotal role played by DCs in generating effector responses, DCs loaded with cancer antigens *ex vivo* are now being investigated for tumour immunotherapy (2-4). Such cellular vaccines are being generated by pulsing DCs with protein, peptides, tumour cell lysates, transfection with genes encoding tumour antigens, and DC-tumour cell hybrids (5, 6). Many clinical trials are ongoing using these approaches for tumour immunotherapy (7-12). Delivery of antigens in particulate form has gained significant attention after observing that DCs had a higher antigen uptake capability when the antigen was delivered in particles as compared to free or soluble form. Since then particulate delivery systems such as polymer-based micro and nanoparticles, liposomes, latex and polystyrene beads have been tested to target model and therapeutic antigens to

DCs for eliciting immune responses. Amongst these different antigen carriers, there has been a rising interest in poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticles over the past decade as it fulfills several requirements for an ideal carrier: PLGA is a biodegradable polymer approved for human use. PLGA particles can be fabricated with various immunomodulators along with the antigen thus morphologically resembling pathogens and triggering the innate immune system and enabling their uptake by APCs. Delivery of antigens in PLGA particles can induce humoral, T helper (Th), and cytotoxic T lymphocyte (CTL) responses.

This thesis explores and evaluates PLGA nanoparticles as antigen delivery vehicles for targeted delivery of antigens and immunomodulators into DCs for the induction of cellular immune responses. The following chapter provides the rationale for targeting vaccines to DCs by discussing the recent advances in DC biology that are of significance in tumour immunotherapy and critical aspects of DCs that are of relevance for developing DC based cancer vaccines. Finally, the PLGA co-polymer based nanoparticles are briefly discussed and their applicability as an antigen delivery system introduced.

1.2 DC origin and phenotype

DCs are a heterogeneous population of cells with a dendritic shape, produced in the bone marrow by differentiation of the precursor cells either through myeloid or lymphoid pathways. These two major subsets are phenotypically and functionally distinct and differ with respect to tissue localization, cytokine secretion, and growth factor requirements (Figure 1-1). In humans, *in vitro* experiments revealed that $CD34^+$ bone marrow progenitors and $CD11c^+$ blood precursors in the presence of granulocyte macrophage

colony stimulating factor (GM-CSF) and interleukin 4 (IL-4) or tumour necrosis factor-alpha (TNF-α) give rise to myeloid DCs (MDCs) (13, 14). MDCs *in vivo* are further sub-classified based on their anatomical location as Langerhans cells (LCs) and interstitial DCs also referred to as dermal DCs. LCs uniquely express CD1a, Birbeck granules, langerin, and E-cadherin (15). Interstitial DCs express coagulation factor XIIIa and are involved in the differentiation of naïve B cells into immunoglobulin secreting plasma cells (16, 17). LCs require transforming growth factor- beta (TGF-β) (18) and are generated from either $CD11c^{+}CD14^{+}$ monocytes or $CD11c^{+}CD14^{-}$ precursors. Interstitial DCs are generated from $CD11c^{+}CD14^{+}$ precursors which can also give rise to macrophages in the presence of macrophage colony stimulating factor (M-CSF) (13, 14, 19, 20). Lymphoid DCs represent the second major subset and are also termed as plasmacytoid DCs (PDCs). *In vivo*, PDCs are derived in the presence of IL-3 (21) from $CD11c^{-}$ blood precursors (22). PDCs are found in the T cell rich areas of the lymph node, spleen, and thymus and prime CD4 and CD8 naïve T cells and secrete IL-12 upon CD40L ligation (22, 23). PDCs are major producers of interferon-alpha (IFN-α) and play a key role in the generation of anti-cancer and anti-viral immune responses (24, 25).

Murine DCs are classified into two main lineages: MDCs (26) and lymphoid DCs (27) (Figure 1-2). Based on their tissue distribution, surface marker expression, function, and expression of CD4 and CD8 molecules, murine DCs can be further classified into six groups. They are $CD4^{+}CD8\alpha^{-}$ and $CD4^{-}CD8\alpha^{+}$ in the spleen and lymph nodes, and $CD4^{-}CD8\alpha^{-/low}$ and $CD4^{-}CD8\alpha^{+}$ in the Thymus. The splenic DC population can be further divided into three types. They are, $CD4^{+}CD8\alpha^{+}DEC205^{+}CD11b^{-}$, $CD4^{+}CD8\alpha^{-}DEC205^{-}CD11b^{+}$, and $CD4^{-}CD8\alpha^{-}DEC205^{-}CD11b^{+}$ which highly express

surface maturation molecules and superiorly activate allogeneic T cells (28). In the lymph nodes, all the above mentioned DC phenotypes of the spleen in addition to $CD4^-CD8\alpha^{low}DEC205^+$ DCs with varying degrees of CD11b expression are present (29, 30). Murine DCs represent an excellent experimental model for deciphering the pathways of DC generation and understanding the mechanisms utilized for generation and maintenance of immune responses. Murine DCs are quite relevant to human DCs in quite a few aspects. Like human DCs, murine DCs also originate from $CD34^+$ bone marrow progenitors or stem cells and are found in blood and peripheral tissues. Murine $CD8\alpha^-$ DCs have been observed to be similar to DC1 while $CD8\alpha^+$ DCs are equated with DC2 (31). Similarly, murine PDC has been described to be similar to human PDC in function (32). Similar to human DCs, murine DCs also take up and process antigens and present them in context with MHC molecules, express co-stimulatory, adhesion, and other accessory molecules involved during T cell interaction, and migrate and mature in response to danger signals.

1.3 Antigen uptake, processing, and presentation

DCs were initially thought to be non-phagocytic and it was believed that macrophages are the key APCs involved in antigen uptake and presentation (33). It is now known that DCs are highly phagocytic during their immature stage. Immature DCs internalize antigen through several pathways. DCs are highly efficient at receptor mediated pincoytosis *via* clathrin-coated pits and fluid phase pincoytosis where the rate of fluid uptake is estimated to be around 1000-1500 μm^3 per hour (34) allowing them to present soluble antigen efficiently at the picomolar range (35). DCs also express cell surface

receptors like mannose receptors (MR) and C-lectin type of mannan binding receptor DEC-205 which mediate receptor mediated endocytosis (34). Other types of receptors expressed on DCs that are involved in antigen uptake include Fcγ receptors (CD32 and CD64), FcεRI and FcεRII, complement receptors (CD11b and CD11c), and the scavenger receptors (αvβ5 and CD36).

Typically, exogenous antigens are processed and loaded onto MHC class II complexes for presentation to $CD4^+$ T cells and endogenous antigens are loaded onto MHC class I complexes for presentation to $CD8^+$ T cells. Exogenous antigens initially enter the early or late acidic endosomal/lysosomal compartments and get degraded by the endosomal proteases. The degraded peptides then associate with the fabricated MHC class II complexes within the MHC class II compartments (MIIC). MHC class II α and β chains are synthesized within the endoplasmic reticulum (ER) and associate with an invariant chain (Ii) which protects the peptide binding groove of the MHC complex from being pre-maturely loaded with cellular self proteins (36). The MHC class II/Ii chain complex is then transported through the Golgi vesicles where it is then localized to the MIIC. Partial proteolytic cleavage of the Ii occurs leaving a small complex called the CLIP (class II associated invariant chain peptide) in the peptide binding groove (37, 38). HLA-DM in humans or the H-2M in the mouse then removes the 'CLIP' allowing the peptide antigen to take its place in the MHC class II complex's groove (39). The cytosomal vacuoles containing the peptide-MHC complex translocate to the periphery and peptide-MHC class II complex gets displayed on the cell surface (Figure 1-3). MHC class II molecules on the surface are again recycled through endocytic pathways and get loaded with new antigens in the MIIC. In the immature stage, the MHC class II-peptide

complexes get accumulated in the lysosomal compartments and do not get displayed on the surface. Upon maturation, MHC class II-peptide complexes accumulate in the nonlysosomal vesicles which migrate to the cell surface. It was also reported that B7 family of co-stimulatory molecules are present in the vesicular lipid along with the MHC class II molecules and are delivered to the cell surface with the peptide-MHC complexes upon activation (40).

Processing of endogenous antigens is through the cytosolic pathway that involves ubiquitination, degradation, and transport by TAP (transporters of antigen presentation) into the ER. DCs initially degrade the cytosolic proteins and with the help of the cytosolic heat shock proteins (HSPs) acting as chaperones transport the formed peptides via the TAP into the ER. In the ER, the peptide gets loaded in the groove of the MHC class I molecule associated with the β-microglobulin (β_2M) (Figure 1-3). Chaperone proteins like the calnexin, calreticulin, and HSP gp96 aid in peptide binding and proper folding of the MHC class I/β_2M complexes and tapsacin aids in the formation of the MHC class I/ β_2M/peptide complexes (41, 42).

Contrary to the above mentioned classical pathway, where exogenous antigen is processed through the MHC class II pathway and endogenous antigen through the MHC class I pathway, exogenous antigens are also processed through the MHC class I pathway by professional APCs. This phenomenon termed 'cross presentation' is a key mechanism for eliciting $CD8^+$ T cells responses against tumours and viral infections and inducing tolerance to certain antigens (43, 44). DCs have been pointed out to be the key cells involved in the cross-presentation phenomena (45, 46). The importance of DCs in crosspresentation was highlighted in one experiment where transgenic mice that

expressed diptheria toxin receptor under a CD11c promoter failed to generate $CD8^+$ T cell responses against exogenous antigens when the mice were treated with diphtheria toxin which selectively depleted $CD11c^+$ cells (47). Few studies have also demonstrated that macrophages are efficient in the cross presentation process (48, 49).

It is widely acknowledged that soluble antigens are inefficient in generating CTL responses *in vivo* and that DCs need to be incubated with large concentrations of soluble antigen *in vitro* for cross presentation (50-52). These studies suggested that soluble antigens enter the cross presentation pathway inefficiently following uptake by fluid phase endocytosis leading to failure for induction of CTL responses. However, soluble particulate antigens generate CTL responses at 1000-10,000 fold lower concentration of soluble antigen (50, 53, 54), which can be inhibited by cytochalasin B, a phagocytosis inhibitor (55). These findings suggested that antigens taken up through phagocytosis were favorably cross-presented by APCs. One reason for the superiority of particulate antigens to generate CTL responses over soluble antigens might be that, higher intracellular concentrations of the antigens are achieved following uptake in the particulate form than soluble forms (56). The mode of antigen uptake can also be pointed to as a determining factor for cross presentation as when the concentration of the internalized antigen taken up either through phagocytosis or endocytosis is kept constant, cross priming was found to be more efficiently performed by APCs that had taken up antigens through phagocytosis (57).

The question that arises is, how are antigens located in the phagosome gaining access to MHC class I presentation pathway. As of this date, two distinct mechansims have been described: one that requires the escape of the antigen from the phagosome to the cytosol

which is classically termed the 'phagosome to cytosol pathway' and the other that occurs in the phagosome itself, termed the 'vacuolar pathway'.

Proteasome inhibitors blocked the cross priming of particulate antigens *in vitro* (55, 58). However, these inhibitors did not affect cross priming of peptides generated endogenously (58), which implied that proteasome inhibitors blocked the generation of peptides from particulate antigens and had no influence on inhibiting the other steps of the MHC class I pathway. The cross presentation of particulate antigens also required the presence of TAP as DCs and macrophages that were deficient in TAP failed to cross-present particulate antigens (58). These findings implied that peptides were generated in the cytosol for particulate antigens and the antigen was translocating from the phagosome to the cytosol. Recent studies have shown that several proteins associated with the ER are also present in the phagosome (59-61). Similarly, Sec61, an ER resident protein involved in the import and export of proteins was detected in the phagosome (62) and was suggested to be involved in the export of proteins out of the phagosome (63). One possible explanation for the presence of ER proteins in the phagosome is that the ER membranes are fusing with the phagosome (64, 65). Also, most of the MHC class I machinery is present in the phagosome. Therefore, there is possibility for the peptides generated in the cytosol from the exported antigens to traffic back into the phagosome through TAP associated on phagosome membranes- 'phagosome to cytosol to phagosome pathway' (61).

The 'vacuolar pathway' has been implicated in cross priming of antigens associated with PLGA particles (66), viral proteins or virus like particles (VLPs) (67, 68), bacterial proteins (69), and even soluble antigens (70). The vacuolar pathway is quite distinct from

the 'phagosome to cyotosol' pathway as it does not require TAP and was found to be insensitive to proteasome inhibitors (71). These observations indicated that the peptides were generated within the phagosome and not in the cytosol in the vacuolar pathway. The classic protease inhibitor- leupeptin was shown to inhibit the vacuolar pathway but had no effect on the 'phagosome to cytosol pathway' (66) which implied that different sets of protease inhibitors operate in the phagosome and the cytosol. Furthermore, DCs and macrophages that were deficient in the protease- Cathepsin S were unable to present antigen by the vacuolar pathway but efficiently presented through the phagosome to cytosol pathway (66), which suggested that Cathepsin S was involved in the vacuolar pathway of cross presentation. So the question that arises is how are the MHC class I molecules acquiring the peptides in the vacuolar pathway. One mechanism that was postulated was that the peptides generated in the phagosomes were regurgitated to the cell surface and were loaded onto empty MHC class I molecules (72). It is also more likely that the peptides are getting loaded onto MHC molecules in the phagosome itself. One possible route is that the MHC class I molecules from the cell surface end up in the phagosome through membrane invagination after phagocytosis. A recent finding that ER fuses with the phagosome (64) suggested the possibility that MHC class I molecules from the ER get translocated to the phagosome through this fusion process.

A third pathway has also been postulated for the cross-presentation of exogenous soluble antigens. This pathway was based on the discovery that soluble antigens are taken up by DCs and transported to the ER (73). From the ER, antigens get effluxed to the cytosol and get degraded and this pathway was termed ER associated degradation

(ERAD) pathway. The cross presentation of soluble antigens has been postulated to occur primarily through the ERAD pathway (74).

1.4 DC maturation

DCs express toll-like receptors (TLRs) on their surface, which recognize distinct molecular patterns associated with the pathogen. TLRs belong to the TLR-IL-1 receptor (TIR) super family and serve as an essential link for DCs to recognize danger signals associated with the host or foreign pathogens by initiating a complex signaling network (75). Ligation of the TLRs triggers a maturation phenomenon that transforms DCs from an immature to mature state. There are 11 known sets of TLRs expressed on DCs that sense different molecules associated with the pathogen (76). In humans, MDCs express TLR1 through TLR5, and also depending on their subcategory, TLR7 or 8, whereas as PDCs express TLR1, 7, and 9 (77-79). TLRs are mainly membrane bound except for TLR9 (that senses unmethylated bacterial CpG) which is present in the ER and moves to the lysosomal compartments (80). Binding of TLRs also transiently augments the endocytic process probably to enhance the antigen capture (81). TLRs are involved in the recruitment of MyD88, TIR domain containing adaptor protein (TIRAP), TIR domain containing adapter inducing IFN-β (TRIF) related adaptor molecule (TRAM), and tripartite motifs (TRIM) all of which are TIR-containing adaptor molecules. Binding of these adaptor molecules to TIRs leads to recruitment of other signaling molecules like Interleukin receptor associated kinase (IRAK) through death domain- death domain interactions which further phosphorylate and activate TNF-receptor associated factors (TRAF6) (82) (Figure 1-4). The end effect of TLR mediated signaling is the release of

NF-kβ and activation of NF-kβ dependent genes like TNF-α, IL-1, and IL-6 (75). MyD88 independent pathways have also been described for TLR signaling leading to DC maturation (83, 84).

Immature DCs have small cytoplasmic process called dendrites or veils on their surface, which provide a large surface area for efficient pathogen uptake. Maturation induces distinct morphological changes in DCs through cytoskeletal reorganization (85) and the dendrites/veils appear to become longer, probably for more efficient interaction with T cells through formation of better T cell synaptic complexes and engaging multiple T cells. One important feature of DCs is their ability to migrate efficiently following maturation. Immature DCs can enter tissues sites through interaction of intercellular adhesion molecule-2 (ICAM-2) with various integrins expressed on the endothelium. DCs also express chemokine receptors (CCR) like CCR1, CCR2, CCR5, and CCR7 that help in the transendothelial migration process. Upon maturation, DCs down regulate the expression of CCR1 and CCR5 and upregulate CCR7 that helps in directing DC migration from the lymphatic vessels to the draining lymph nodes through interaction with MIP-3β (CCL19) and secondary lymphoid tissue chemokine (SLC or CCL21) (86, 87). Other chemokines that are secreted by mature and activated DCs include Interferon inducible protein (IP-10) and thymus and activation- regulated chemokine (TARC), which help in recruiting various T cell subsets and regulated on activation normal T cell expressed and secreted (RANTES), macrophage inflammatory protein-alpha (MIP-1α), and MIP-1β that help in recruiting monocytes and other DCs to the local environment. Mature DCs also express a number of characteristic markers like CD83 which is involved in $CD4^+$ T cell development and cell to cell interactions (88, 89) and DC- lysozyme

associated membrane protein (DC-LAMP), a DC specific lysosomal protein (90). Furthermore, mature DCs upregulate their expression of MHC class II and co-stimulatory and accessory molecules like CD80, CD86, CD58, CD54, CD50, and CD40. The upregulation of these molecules leads to bidirectional signaling between DCs and T cells like enhancement in signal 1 via MHC and T cell receptor, signal 2 through B7-1/B7-2 and CD28, and signal 3 which induces cytokine secretion and T cell responses (91).

Various stimuli induce the maturation process within DCs. The source of the maturation stimuli can be from host-derived factors like inflammatory proteins, CD40L, TNF-α, IL-1, IL-6, and IFN-α or molecules released by the damaged host tissues or pathogen associated products derived from microbial infections. *In vitro* maturation of DCs can be achieved by lipopolysaccharide (LPS), double stranded ribonucleic acid (dsRNA), apoptotic cells, immune complexes, Cytidine phosphate guanine-DNA (CpG-DNA), proinflammatory cytokines, and prostaglandins (92, 93).

1.5 DC interactions with lymphocytes

The interaction of DCs with T cells is short lived and antigen independent (94). The spleen and the lymph node represent the two major sites where priming of T cells by DCs occurs. There are three distinct phases during T cell priming by DCs: the initial interaction of a single T cell with many DCs, a sustained T cell-DC interaction lasting for up to several hours during which naïve antigen specific T cells are transformed into cytokine secreting effector T cells, and finally dissociation of the DC-T cell complex and clonal expansion of the activated T cells before exiting the lymphoid organ. A recent study using *in vitro* real-time imaging of lymph nodes revealed that a single DC can

interact with as many as 500 T cells in one hour (95, 96). In presence of antigen, long lasting stable DC-T cell complexes are formed (95-98) during which a single DC can simultaneously engage at least 10 T cells. There have been reports that Rho family guanosine triphosphatases 1 and 2 (Rac1 and Rac2) expressed in mature DCs control the membrane ruffling and formation of dendrites and cytoskeletal protrusions of these dendrites towards T cells for effective T cell engagement (99). In addition, they have also been implicated in DC migration towards T cells.

The nature of a T cell response elicited following priming by DCs is dependent on several factors like, concentration of antigen on DC, maturation stage of the DC and the type of maturation stimuli recieved, binding affinity of the T cell receptor for the peptide-MHC complex (pMHC) and the duration of the DC-T cell interaction (100). Mature DCs are also required for effective T cell stimulation as they form well organized immune synapses with T cells with long lasting interactions. In contrast, immature DCs form less stable conjugates with T cells which are short lasting and intermittent with no distinct organization leading to improper T cell activation (101). Mature DC priming also generates T cells with enhanced T cell survival capacity, also referred to as "T cell fitness" (100). The enhanced T cell survival capacity is due to their induced responsiveness to IL-7 and IL-15, the two key survival cytokines that help in sustaining cellular metabolism (100).

DCs prime $CD4^+$ T cells and skew the immune responses either towards Th1 or Th2 types or induce regulatory T cell subsets (102). Th1 immune responses are elicited following secretion of IFN-γ by activated $CD4^+$ T cells that support generation of $CD8^+$ T cells responses. Th2 immune responses are generated following secretion of IL-4, IL-5,

and IL-13 by the activated $CD4^+$ T cells. The polarization of immune responses towards the Th1 or Th2 type after DC stimulation also depends on the cytokine secretion profile of DCs at the DC-T cell microenvironment. Secretion of IL-12, IL-18, and IL-27 by DCs leads to the generation of Th1 responses whereas absence of these cytokines induces Th2 responses. The type of TLR ligand used for DC maturation also influences the nature of the ensuing immune responses (103). LPS and bacterial flagellin, which are TLR4 and TLR5 agonists respectively, induce the secretion of IL-12 by DCs leading to generation of Th1 responses. In contrast, PamScys, a TLR2 agonist suppresses IL-12 production thereby inducing Th2 responses.

1.6 DC based cellular vaccines for cancer immunotherapy

1.6.1 Why adoptive DC therapy?

DCs are being considered for cancer immunotherapy for several reasons. DCs are responsible for the recognition and uptake of TAA and migrating to the tumour draining lymph node for presentation of the tumour antigen derived peptides to T cells leading to initiation of CTL responses, which can effectively clear the tumour cell. However, there are several obstacles in cancerous conditions that limit the effectiveness of DCs to elicit successful anti cancer immune responses.

1.6.1.1 Reduced DC numbers

In a normal healthy individual, DCs represent less than 0.1% of the total leukocyte population present in the body. However, in spite of their extremely small numbers, DCs possess the unique capability to interact with T lymphocytes very effectively and prime

them in a very efficient manner. One of the classical examples of their effectiveness is the ability of a single DC to interact with more than 500 T cells in 1 h (95). However, even this extremely low numbers of DCs is even more reduced in cancer (104-106). The physiological consequence of this diminished population of DCs results in lesser numbers of DCs available for infiltrating the tumour environment. In this line, there are several reports of inefficient recruitment of DCs to tumour sites leading to poorer prognosis of patients with various types of cancer.

1.6.1.2 Altered differentiation and maturation of DCs

Defective maturation status of DCs has been observed in tumour bearing animal models and in cancer patients (107-109), which indicated that the DCs in the tumours environment are immunosuppressed. Similarly, DCs isolated from patients with breast, head and neck cancers had reduced ability to induce allogeneic and antigen specific T cell responses (110, 111). On closer examination, these DCs had reduced number of MHC and co-stimulatory molecule expression as compared to the normal pattern seen in mature DCs. Similarly, DCs obtained from tumour bearing mice were unable to generate CTL responses and expressed lower number of MHC class II, CD86, and adhesion molecules (112). Tumours and tumour-derived factors have been implicated to play a key role in conditioning the DC to be in their immature stage as a strategy to evade immune attack. In one study, tumour-derived stromal-derived factor (SDF)-1 in ovarian cancer patients recruited DCs but maintained them in an immature state (113). Thus inhibitory factors that are derived from tumours affected DC differention leading to impaired DC function. Current literature confirms that tumours directly or indirectly induce the release of several factors, mainly IL-6, IL-10, M-CSF, vascular endothelial growth factor (VEGF),

gangliosdies, and prostanoids that directly affect DCs and retain them in an immature state (114-118). IL-10 has been implicated in impairing the differentiation of monocytes into DCs and inhibited the DCs ability to stimulate antigen specific T cells and more importantly induced T cell anergy (119). IL-10 also suppressed the expression of maturation molecules on DCs which in turn exhibited tolerogenic functions (120). IL-10 thus aids tumour evasion through inhibiting DC differentiation and maturation. Similarly, IL-6, secreted by tumours affects DC differentiation from $CD34^+$ progenitors through modulating the expression of GM-CSF receptors (115). The expansion of DC progenitors was also found to be severly impaired by IL-6 exposure (121). Also, IL-6 has been implicated in inhibiting DC differentiation by maintaining them in their immature stage (122). The cytokine, GM-CSF has also been reported to play a significant role in tumour evasion. GM-CSF is produced by several types of cancer cells and aids in cancer cell metastases (123, 124). However, GM-CSF has been tested to be beneficial in inducing anti cancer immune responses when cancer cells were made to express this cytokine (125). One possible reason for this paradigm was, aberrant production of this cytokine is detrimental for the generation of an effective immune response as overproduction of GM-CSF by tumour cells suppressed CTL responses through maintaining DCs in an immature stage (126). Similarly, VEGF was found not only to be involved in angiogenesis through aiding the proliferation of endothelial cells that form vasculature for the growing tumour but also in inhibiting the production of DCs from hematopoietic stem cells (127). Increase in the levels of VEGF has been associated with poor prognosis of cancer patients (128). Similarly ganglisodes have been implicated to aid in tumour growth through inhibiting the differention of hematopoietic progenitors into DCs (129).

In view of the evidence for the defective nature of DCs in cancer patients, a strategic approach for tumour immunotherapy is to generate and condition DCs *ex vivo* as a means to bypass tumour induced suppression *in vivo*. In this regard, DCs propagated *in vitro* from hematopietic preogenitors obtained from cancer patients were found to be 'immuno-potent' whereas circulating DCs obtained from the same cancer patients remained defective. DCs can be generated in large numbers from peripheral blood monocytes or bone marrow progenitors of cancer patients. A number of protocols have been developed and robustly validated to generate DCs *in vitro* (discussed later in this chapter). The DCs generated under these conditions are not immunologically compromised due to their propagation in the absence of the immunosuppressive environment.

Generation of DCs *ex vivo* also has the advantage of selective tumour antigen targeting. Direct administration of tumour antigens to patient limits the effectiveness of the vaccination strategy as the administered antigen can get diluted in the physiological milieu limiting its therapeutic concentration needed for the generation of immune responses. Also, the administered antigens need to be taken up by professional APCs, mainly the DCs. However, it is highly unlikely that an appreciable number of resident DCs take up the administered antigen as the number of DCs available *in vivo* is extremely low. In addition, the administered antigen may not always reach the DCs due to uptake by other cell types and degradation of the antigen in the harsh *in vivo* physiological milieu. Generation of DCs *ex vivo* can thus circumvent the situation of less number of available DCs *in vivo*. In addition, the phenotype, particularly the maturation stage of the DCs can be controlled such that antigens can be fed to DCs in their immature stage. Also, *ex vivo* pulsing of the antigen assures that the antigen is being taken up by DCs and

not any other cell type and the antigen is a biologically active form due to the absence of degrading enzymes in the culture media.

In this respect, there are numerous preclinical and clinical studies where autologous DCs generated and expanded *ex vivo* from progenitor cells and loaded with the tumour antigens and introduced *in vivo* generated CTL responses, regressed existing tumours and protected the animal against tumour cell challenge (130-133). Thus, the approach of generating functional DCs from rare precursor cells *ex vivo*, followed by tumour antigen loading and maturation, and reinfusion back to the patient is widely being investigated for tumour immunotherapy.

1.6.2 DC culture for tumour immunotherapy

Ever since DCs have been recognized for their potential in the generation and maintenance of immune responses, they have been widely investigated as important components of several therapeutic vaccination strategies for cancer and infectious diseases. Initial protocols for generating DCs focused on negative selection using antibodies and magnetic beads. However, DC yield following negative selection was very minimal due to their presence at very low numbers in the body. Advances in generating DCs from monocytes in the presence of growth factors led to the development of various DC generation protocols that paved way for current DC vaccination studies.

Peripheral blood monocytes are the largest source of DC precursors and can be differentiated into immature DCs in the presence of GM-CSF and IL-4. Monocytes are currently being isolated by quite a few protocols and some of them include- positive selection, counterflow elutriation, and culture plate adherence (134). However, one of the

key limiting factors in generating DCs from monocytes *ex vivo* is endotoxin contamination. To circumvent this obstacle, closed culture systems have been devised using culture bags and cell factories (135, 136). Automated devices that directly isolate DC precursors into sterile tissue culture bags through positive selection have also been tested (137). In one protocol, unfractionated mononuclear cells were transferred directly into culture bags containing GM-CSF and IL-13 to minimize physical manipulation. Several comparative studies have been performed to address the clinical significance of DCs generated by different isolation and propagation procedures (138, 139). However, a standard protocol for DC generation still remains unaddressed due to variation in culture conditions used for the generation of clinical grade DCs by various investigators. Though early studies used freshly cultured DCs, many recent reports demonstrate that immature DCs and immature and mature antigen loaded DCs can be frozen and thawed prior to use without compromising their viability or immune effector function (140, 141). Application of these cyropreservation methods for clinical DC vaccination can significantly minimize the influence of culture conditions and other experimental variables that can impact the therapeutic outcome of the DC vaccination protocols.

1.6.3 Nature of antigens loading DCs

In light of the described functional characteristics of DCs, antigens need to be targeted to this cell type in order to fully exploit its unique processing and presentation machinery. Proteins can be used as a source of tumour antigen either as purified form or as a recombinant preparations from DNA (142). The advantage of using protein antigens is the possibility to select an array of immunogenic epitopes so that no pre-selection or

screening of the peptides is necessary (143). In addition, the APC machinery can process the protein antigen into peptide epitopes that can be loaded onto their MHC molecules circumventing the need for selection of the haplotype based on MHC alleles. Thus, delivery of protein antigens containing both the classes of epitopes offers the scope of inducing immune responses against a variety of epitiopes assosciated with the protein antigen thereby minimizing the chances of tumour escape. Protein antigens also have a few disadvantages. Purification of proteins from natural sources can be accompanied by accidental contamination that poses a significant risk for clinical application. Protein antigens if used for selective *in vivo* DC targeting are rapidly degraded by proteases thereby limiting their therapeutic significance. Proteins are also unstable during storage, *in vivo* hydration, and even incubation at 37°C (144).

Tumour peptide antigens have been widely employed for DC loading (145-147) and such DCs are effective in generartion of CD4 and CTL responses (148). Peptide antigens can be generated by synthetic methods circumventing the need to have a tumour source. Furthermore, synthetic methods of peptide synthesis curtails the need for a biological system for vaccine production (149), thus allowing development of safer vaccines. From a pharmaceutical perspective, analysis, quality control, and scale up for production are straight forward for peptides (149). The risk of autoimmunity is also minimized by limiting the use of peptides with tumour-specific mutations, expressed only in the tumours and not in normal tissues. The immune responses can also be precisely monitored due to their well defined epitopes although epitope spreading has been reported even when single peptide antigens were employed for vaccination (150, 151), probably due to presentation of tumour antigens obtained from lysed tumour cells

following CTL mediated killing. Peptide vaccines can also be designed to elicit an immune response against a select or panel of peptides giving the feasibility to control the type of immune response. The limitations to using peptides include their restricted antigenic repertoire, need for prior knowledge of the tumour-specific epitope, limited immunogenicity, and MHC restriction. Peptides eluted from autologous tumour cells represent an alternative source for obtaining MHC class I and II restricted peptides for use in DC loading (152, 153). MHC restriction can also be countered by careful selection of peptide antigens and delivery of multiple epitopes in one vaccine.

Whole tumour cell vaccines were developed with a view to deliver an extensive range of tumour cell derived epitopes to DCs for the generation of polyclonal immune responses. These broad range of immune responses directed against various target antigens expressed on tumour cells reduce the possibility of tumour cell escape that is commonly observed when single antigens are employed for tumour immunotherapy (154). Some of the preparations using this strategy include whole tumour cell lysates (131), apoptotic or necrotic tumour cells (155), and DC-tumour cell hybrids (156). Delivery of whole tumour antigens to DCs circumvents the need to have well defined tumour antigens. The applicability of this vaccine depends upon the availability of the tumour antigen and also on the concentration of the tumour antigen during loading onto DCs. The simplicity of using tumour lysates for DC pulsing is quite attractive as fresh preparations of the tumour are no longer needed as tumour lysates can be prepared in advance and frozen and thawed prior to DC loading (157). A study that involved *ex vivo* pulsing of monocyte derived DCs with whole tumour cell preparation followed by reinfusion into cancer patients generated mixed responses including complete remission,

partial response, stable disease and progressive disease (158). Irradiated tumour cell-DC vaccine has also been investigated for the immunotherapy of metastatic melanoma and has given promising results in cancer patients by lowering the tumour burden (159). Tumour cell-DC hybrids have also been devised (156) allowing the direct targeting of tumour antigens and endogenous proteins for processing through the MHC class I pathway. This procedure is however laborious due to selection of viable and fused cells which need further expansion. Although, therapeutic immune responses have been observed with this hybrid system, the effect of the fusion process on DC functionality remains to be answered (160). One of the key limiting factors for using whole tumour cell preparations for DC targeting is the induction of autoimmunity against self epitopes, which are expressed by tumour cells and normal cells. Finally, as a repertoire of tumour antigens are used in this strategy, evaluation of immune responses becomes complex and cumbersome.

Tumour antigens in the form of genetic material comprising of either DNA or RNA is also one of the key avenues that is gaining increasing importance in developing DC based tumour immunotherapy protocols. Total RNA, amplified RNA, or tumour antigen specific RNA can be employed as the source of whole tumour cell antigen for loading onto DCs for the generation of polyclonal immune responses (161). The advantage of using RNA as the source of tumour antigen is that, sufficient amounts of clinical grade tumour antigen can be prepared by reverse transcription and polymerase chain reaction (RT-PCR) (162, 163). DCs transfected with tumour antigen RNA were found to be potent in inducing CTL immune responses (164, 165). In addition, RNA transfected DCs have been found to be as effective as tumour lysate pulsed DCs in inducing polyclonal immune

responses and were also more superior to peptide targeted DCs in generating anti tumour immune responses (161, 166, 167). One of the other advantages of using RNA as the tumour antigen source includes easier generation of total tumour RNA as compared to generation of cDNA libraries from tumours. The disadvantages include greater degree of manipulation, lesser efficiency of transfection, and lower amount of translated protein detected in the cytoplasm. Transfection of DCs with DNA encoding tumour antigens allows the endogenous expression of these encoded antigens allowing for processing on both the MHC class I and MHC class II pathways.

One more avenue that is of interest is the direct injection of *ex vivo* generated DCs into the tumour site. As tumour cells are the source of the antigen, this approach offers the feasibility for DCs to select the predominant antigens from apoptotic and necrotic tumour cells. In one study, unloaded DCs were injected intra-tumourally into implanted gliomas in rats and tumour regression with an acceptable degree of success was observed with tumour infiltrating lymphocytes detected in the regressing tumours (168). These studies suggested that DCs generated therapeutic immune responses by picking up tumour antigens even in a microenvironment that strongly inhibits development of tumour specific immune responses (169). Combinational therapies that involved intra-tumoural administration of unloaded DCs into tumour bearing mice that had undergone either radiation or chemotherapy resulted in complete elimination of the tumour whereas tumour bearing mice that have not undergone these protocols, but just received the DCs showed no inhibition of tumour growth (170, 171). These studies indicated that combinational therapies involving radiation and chemotherapy destroy the tumour leaving behind apoptotic and necrotic cells and the administration of unloaded DCs pick

up these cells and generate immune responses that ultimately clear the remaining tumour cells.

1.6.4 Strategies to deliver antigens into DCs

Antigens have been introduced into DCs using various approaches. The following paragraphs focus more on the newer approaches that are being investigated to introduce antigens into DCs besides the classical approach of soluble antigen targeting that utilizes macropinocytosis for antigen uptake (52).

1.6.4.1 Receptor mediated antigen loading

APCs express surface receptors that specifically bind the Fcγ portion of Ig. Therefore, conjugation of antigens to antibodies allows for the antigen to be targeted to DCs through Fc receptors (FcR) (172-174). This mode of antigen delivery efficiently target antigens into DCs and generated MHC class I responses. More recently fusion proteins have been introduced where the construct comprises of a protein antigen and a FcR restricted monoclonal antibody (175). The selectivity for Fcγ binding is critical for MHC class I presentation as targeting through Foε portion induced MHC class II restricted responses (176). In addition to FcR mediated antigen delivery, several other surface receptors expressed on DCs have been explored to introduce antigens into DCs. These include MR, DEC-205, and the Hsp receptor- CD91 (177). Targeting antigens through these receptors induced mixed immune responses. Antigen targeting through MR and DEC-205 primarily induced MHC class II restricted responses (34, 178, 179) whereas Hsp receptor targeting induced DC maturation (180-182) and generated MHC class I restricted responses (177, 183-186).

1.6.4.2 Particulate antigen delivery systems

Antigens have been targeted into DCs in particulate form based on the superior ability of DCs to take up particles. Immense interest in particulate antigen delivery began after DCs were observed to take up antigens as particles 1000 times more efficiently than soluble antigens. Many different kinds of partices have been investigated to introduce antigens into DCs and some of the key delivery systems are discussed below.

1.6.4.2.1 Non-infectious viral sub-unit vaccines

Also called VLPs or virosomes, they are generated in mammalian and insect cell expression systems. Viruses like human immunodefiency virus (HIV), parvoviruses (PV), and hepatitis B (Hep B) have the unique ability to assemble into particles giving the possibility to entrap antigens into their shells. In one study, PV was found to mature DCs in the absence of any adjuvant and induced the secretion of pro-inflammatory cytokines in macrophages and DCs (187). Both, CD4 and CD8 T cell responses were generated in mice vaccinated with VLPs carrying capsid protein indicating that this mode of antigen delivery is efficient in cross-presentation (188, 189).

1.6.4.2.2 Biodegradable particles

Antigens encapsulated in polymeric micro or nanoparticles is a new approach where the antigen is protected from degradation and is released in a sustained manner over a period of time (190). Many of these particles have been synthesized using poly (D,L-lactic-co-glycolic acid) (PLGA) copolymer, poly(ortho esters) (POE), and immunostimulating complexes (ISCOMs).

PLGA is a biocompatible and biodegradable polymer approved for human use and has been extensively studied to fabricate micro and nanoparticles. Both macrophages and

DCs have been found to efficiently internalize PLGA particles *in vitro* (191). DCs targeted with PLGA microparticles containing malarial and flu associated antigens generated potent $CD8^+$ T cell responses *in vitro* (192). Sustained T cell activation was also observed following delivery of antigens entrapped in PLGA particles to DCs and this phenomena was attributed to controlled release of the antigen from the particles inside DCs (192). Cytoplasmic delivery of the entrapped antigen is also achieved inside the DCs following uptake of PLGA particles (55, 193, 194). More recently, DCs pulsed with antigen encapsulated in PLGA particles *in vitro* generated CTL responses *in vivo* far more efficiently than delivery of the antigen to DCs in the soluble form (195). Thus PLGA mediated antigen delivery to DCs dramatically enhaces antigen presentation by both the MHC class I and II molecules and thus holds immense potential for clinical applications. POE particles encapsulating plasmids were found to efficiently induce cellular and humoral immune responses in mice and even protected the mice following subsequent challenge with tumour cells (196).

Liposomes are phospholipid spheres that have been investigated as antigen delivery vehicles for bacterial and viral infections and tumours (197-199). Liposomes similar to PLGA particles, protect the antigen from degradation and prolong the immune responses associated with the antigen and mainly generate MHC class I restricted immune responses (200). Many studies report that DCs loaded with antigens entrapped in liposomes generate efficient MHC class I responses (201-203). Murine DCs pulsed wth OVA containing IgG liposomes *in vitro* were found to prevent the growth of OVA expressing tumour *in vivo* at a prophylactic and therapeutic level (204). Similarly, human DCs loaded with liposomes containing tumour lysate activated immunosuppressed

tumour infiltrating lymphocytes (TILs) through induction of CTL responses (205). One potential pharmaceutical limitation for application of the liposomes is their relative instability and route of adminsitration.

ISCOMs are approximately 40 nm diameter matrix constructs comprising of antigen, cholesterol, saponin, and phospholipids (206). Their small particle size and lipophilic nature allows ISCOMs to integrate with cell membranes and promote uptake (207). DCs pulsed *in vitro* with ISCOMs containing model antigen OVA induced proliferation of naïve $CD4^+$ T cells (208). In addition, mucosal immunity in mice has been observed following administration of ISCOMs by oral, intranasal, parenteral, and intravaginal routes making this delivery system very versatile for administration (209). However, one disadvantage with ISCOMs is that formulation is difficult to prepare and requires extensive antigen modifications and reformulation (210).

1.6.4.2.3 Non-degradable particles

Particles in this category include latex, gold, silica, and polystyrene particles. Long term immune responses have been observed with these carriers possibly due to their retention at the injection site or tissues. In one study, antigen coupled to latex particles induced MHC class I responses more efficiently than soluble antigen (211). Similarly, macrophages that internalized antigen adsorbed onto latex beads presented the antigen 1000-10,000 fold more efficiently than soluble antigen for MHC class I presentation (50) or MHC class II presentation (212). Similarly, polystyrene particles have been reported to introduce antigens into DCs (213, 214). In one recent study, polystyrene particles containing OVA were efficiently taken up by DCs *in vitro* and these DCs activated antigen specific T cells in a superior fashion than free antigen delivery (215). In another

study that did not involve antigen delivery, antihuman CD40 antibody conjugated onto polystyrene nanoparticles superiorly matured human DCs *in vitro* (216). The main disadvantage of using non-degradable material is that the delivery system can pose significant toxicity issues. More conclusive studies directed at assessing long-term toxic effects of these nanoparticles on DCs as well as on select tissues need to be addressed.

1.6.4.2.4 Viral vector mediated antigen delivery

Genetically modified recombinant viruses are highly efficient vectors for the delivery of DNA into DCs for subsequent antigen expression. A variety of viruses have been manipulated to serve as vehicles for introducing the DNA into DCs. These include retrovirus (217), adenovirus (218), herpes simplex virus (219), vaccinia virus (220), influenza virus (221), and alpha virus (222). Amongst these, transduction efficiencies of vectors derived from adenovirus, herpes simplex virus, and influenza virus were as high as 90% (219, 221, 223) making them an ideal choice to introduce tumour DNA into DCs. Replication-incompetent retroviral vectors encoding tumour antigens have been employed for antigen delivery to DCs. Retroviruses offer unique advantages like stable integration into the target cell genome allowing for generation of full length proteins that provide lasting and enhanced tumour antigen presentation. Also, as viral structural proteins are not produced due to the removal of the *gag*, *pol*, and *env* regions, immune responses towards these structural sequences are not seen. Some of the limitations of using retroviral vectors are their low transduction efficiency (224) and their integration into dividing cells limiting their applicability to DCs obtained from the peripheral blood or generated *in vitro* which are non-replicating. A few approaches of retroviral

transduction of non-dividing DCs have been reported (225, 226) as well as transduction of replicating $CD34^+$ progenitors which are differentiated into DCs (227).

Adenoviruses are DNA viruses made replication incompetent by removal of the E1 gene and are capable of integrating into non-dividing cells and represent one of the most potent vectors for gene transfer to DCs (228). The advantages of using adenovirus as gene delivery vectors are their ease of large scale production, accommodation of large gene constructs (up to 7.5 kb), and high transduction efficiencies without compromising the DC functionality (223). One of the limitations in using adenoviruses in the generation of host immune responses against the vector due to transcription of viral genes encoded by the protein that can result in clearance of the vector. However, transduction of DCs with adenoviruses followed by their vaccination was found to be relatively safe as many studies have reported the beneficial effect of this vaccine without compromising its applicability. In one study, multiple immunizations of the DC-viral vaccine induced antigen specific immune responses that were not affected when neutralizing antibodies against the adenovirus were used (229). In another study, vaccination with adenovirus transduced DCs induced antigen specific immune responses in mice pre-immunized against adenovirus (230). To further reduce the non specific immune responses, adenovirus particles were irradiated to inhibit transcription of structural proteins and were still found to possess high infection rates to DCs leading to efficient gene transfer (231). Also, many studies have demonstrated the applicability of adenoviruses as efficient transducers of DCs for generation of tumour specific immune responses (232-235). Furthermore, adenovirus mediated transfection of DCs was found to generate more potent tumour protective immune responses than peptide pulsed DCs (236).

1.6.5 Polarized DC maturation

Antigen loading into DCs does not necessarily bring them to a mature state. Maturation of DCs is achieved following incubation with cocktail of pro-inflammatory cytokines like IL-1β, TNF-α, and prostaglandin E_2 (PGE_2) (92). The manner in which the DCs secrete cytokines changes considerably based on the type of maturation stimulus provided i.e. recipe of the cytokine cocktail. Altering the cytokine cocktail can dictate and polarize the DCs to a particular maturation phenotype (91). DC maturation achieved following incubation with poly-I:C and IFN-γ generated type 1 polarized DCs or DC1 that induced potent melanoma specific CTL responses after a single *in vitro* sensitization of T cells obtained from patients with melanoma. Furthermore, CD4 T cells stimulated by DC1 produced IFN-γ, which indicated that the induction of Th1 type responses was mediated through DC1. Similarly, reversal of existing Th2 immune responses to Th1 response was also possible through engineered DC1s admixed with exogenous IL-12 (237).

The ability of DCs to direct polarized immune responses towards either Th1 or Th2 type most importantly determines their utility for DC therapy. Functionally mature DCs needs to prime and expand CD4 and CD8 T cells to promote tumour regression and sustain a long term disease free condition. Thus, the procedures involved in generating functionally mature DCs to induce DC polarization is critical for the generation of therapeutically effective DCs. A better definition of mature DCs need to established, as till date no specific criteria of the appropriate functional characteristics of mature DCs are available. The generally acceptable notion that DCs generated *ex vivo* should have a 90%

viability and be able to produce either Th1 or Th2 promoting cytokines is clearly not sufficient and adequate.

1.6.6 Vaccine administration

One of the key aspects to be considered for the success of DC vaccines is the appropriate route of vaccination as the administered DCs should be capable of trafficking and localizing to the lymphatic tissues. The route chosen should be suitable enough to ensure induction of maximal immunogenicity (238). In addition, the number of administered DCs and their maturation status play a key role in determining the interaction of these DCs with other cell types *in vivo*. The migration time of the administered DCs to reach the lymph nodes from the site of vaccination is also important for peptide loaded DCs which have a very limited half life of the pMHC complexes (239). DC based vaccines are usually administered through the subcutaneous (s.c), intradermal (i.d), intravenous (i.v), and intraperitoneal (i.p) routes. For DC vaccines directed for selective targeting to the T cell areas of the lymph nodes, murine studies have demonstrated that s.c. route of vaccination was superior to the i.v. route (240). Similarly, studies in chimpanzees have also shown that DCs administered through the s.c. route are localized in the draining lymph nodes where they are detected for up to 5 days (241, 242). However, some human studies have shown that regional lymph node targeting could only be achieved following vaccination of DCs through the i.d. route (145, 238). With regard to the generation of immune responses, Th1 type immune responses were predominantly generated following administration of DCs by the i.d. and the intralymphatic (i.l.) routes whereas i.v. route of administration generated humoral immune responses (243). The

delivery of DCs through the i.l. or the intranodal (i.n.) routes allows for their direct localization into the lymph nodes thereby increasing the efficacy of the vaccination approach. The i.l and i.n routes thus offer the advantage of minimizing the number of DCs being administered and enhance the chance of higher DC localization to the T cell areas of the lymph nodes.

The dosing and frequency of administration of DCs for clinical trails are based on observations following their vaccination in to animals and human subjects. The most widely followed dosing interval is the weekly to monthly administration of DCs. The impact of the number DCs and their frequency of administration are undoubtedly the most critical parameters affecting the induction of T cell responses. One study reported a diminished CTL response following multiple weekly vaccinations of DCs (244). The diminished responses could be due to clonal exhaustion of the responding T cells and induction of T cell tolerance following multiple DC vaccinations.

1.6.7 Evaluation of DC immunotherapies

As DC vaccines are targeted at modulating the immune responses, suitable approaches that are reliable and reproducible are needed for monitoring these immune responses and to confirm that the observed effects are being mediated by DCs (245). A few of the most widely employed immunoassays are enzyme-linked immunospot (ELISpot) (246), colony forming cell assay (CFC) (247), tetramer analysis (248), and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) (249). Also, tumour specific endpoints like delayed type hypersensitivity (DTH) to tumour antigens and reduction in tumour burden are used. One of the main parameters to assess the functionality of the vaccination

is the CTL activity and cytotoxic granule release. These methods are powerful enough to enumerate qualitative and quantitative responses of the responding T cells generated against the tumour. It is also advisable to use more than one assay for assessing the efficacy of the vaccination.

To analyze the effector responses generated following DC vaccination, immune cells can be directly isolated from the blood, lymphoid organs or from the tumour site. Assessment of the immune responses at the target site can provide critical information regarding the condition of the ongoing immune responses (250). The outcome and significance of the treatment regimen can also be enumerated by freezing peripheral blood mononuclear cells (PBMCs) of the patient at different time points during therapy and analyzing all the samples at once, which would limit inter-assay variability. However, care must be taken during handling as the quality of the assay can be largely influenced by the manner in which the cells are frozen (251-253).

As CTL's play a predominant role in tumor regression, the efficacy of cancer vaccines is monitored based on their potential to induce cellular immune responses. However, measurement of tumour specific IgG type antibody response can aso be employed to monitor the success of DC vaccines. Generation of antibody response to HER-2/neu and carcinoembryonic antigen (CEA) has been found to be effective for the development of a therapeutic response (254, 255). A strong correleation between increase in survival time and generation of vaccine induced antibody responses has been observed in melanoma patients (256-258). Thus, it would be ideal to include the assessment of tumour specific antibody responses as one of the end point parameters measured in DC immunotherapy studies for cancer (259).

1.7 Poly(D,L-lactic-co-glycolic acid) nanoparticles as antigen delivery system

PLGA is a food & drug administration (FDA) approved biodegradable polymer that has been used in resorbable surgical sutures (260). The first application of the PLGA for microencapsulation of vaccine antigens occurred in 1979 which revealed that these biodegradable nanoparticles were capable of releasing the antigen at a sustained rate and generated humoral immune responses (261). PLGA is now being widely studied for controlled release of drug delivery systems (262, 263). It is useful in the formulation of a variety of molecules having different chemical properties. The application of PLGA nanoparticles as antigen delivery systems combines the controlled release properties of a polymeric system with the ease of administration. A number of research groups have encapsulated proteins, peptides, plasmid DNA, oligonucleotides, viruses, and a variety of immunomodulatory molecules using this biodegradable polymer (264-271). Hydrophobic molecules such as lipopeptides and glycolipids can be formulated into PLGA particles as single emulsion (oil/water) formulation whereas as hydrophilic molecules can be prepared as double emulsion (water/oil/water) formulations.

Formulating protein and peptide antigens in PLGA nanoparticles poses several challenges. During the formulation of the antigen containing nanoparticles, contact with organic solvent, water-oil interphases and lyophilization can denature the antigen, a major issue for protein antigen delivery for the generation of antibody responses. Furthermore, exposure of the encapsulated antigen to the acidic pH during polymer degradation is another factor that can degrade the antigen. In comparison of liposomes to PLGA nanoparticles, liposomes are prepared under milder conditions than PLGA nanoparticles (272). However, the potential drawbacks of liposomes is their relatively

instability where aggregation and leakage of the entrapped antigen poses a hurdle for long term storage (272). Furthermore, scale up for bulk pharmaceutical production is a tedious process. In comparison to liposomes, PLGA nanoparticles maintain the antigen in a dry state whereas the former requires aqueous environment to maintain the formulation characteristics (272). The formulation of antigens as dry vaccines using PLGA nanoparticles further simplifies the downstream storage process thus making it a versatile candidate for rapid immunization programs, especially in developing countries.

1.7.1 Composition and release

PLGA is a co-polymer made up of lactic acid and glycolic acid monomers linked together through ester bonds (Figure 1-5). Particles fabricated with the PLGA copolymer are monolithic where the hydrophilic or hydrophobic antigen is homogenously dispersed in a matrix of the polymer. Degradation of the polymer occurs through non-enzymatic hydrolysis of the backbone ester linkages giving rise to lactic and glycolic acids (Figure 1-5). Therefore, manipulating the composition of the lactic:glycolic acid ratios and molecular weight of the polymer can give PLGA nanoparticles with the desired characteristics of hydrolysis and antigen release rates (273-275). The lactic to glycolic acid composition of the co-polymer also affects the crystallinity, hydrophobicity, and the degree of hydration of the polymer. A 50:50 ratio of the lactic to glycolic acid gives a higher rate of degradation than polymers fabricated with higher proportions of either of the monomers due to the amorphous nature of the polymer. To generate PLGA co-polymers with slower lower degradation characteristics, the composition of the lactide acid is increased due to its higher hydrophobicity than glycolic acid. Also, the D,L lactic

acid is preferred over L-lactic acid as it forms an amorphous polymer where in the antigen or the drug is homogeneously dispersed within the polymeric matrix (272). The release profile of antigens from PLGA nanoparticles is triphasic with an initial quick burst release followed by a lag phase and then continued /zero-order release due to bulk erosion of the polymer (276, 277).

1.7.2 PLGA nanoparticle uptake by DCs and macrophages

Most of the initial investigation on uptake of PLGA particles focused on macrophages (278-280). The uptake of these particles is affected by size, surface charge, and hydrophobicity. Due to the superior role played by DCs in generating and maintaining immune responses, there has been a growing interest in assessing the ability of DCs to uptake PLGA nanoparticle (191, 192, 216, 281-289). It was also observed that DCs were the key players in the uptake of PLGA nanoparticles *in vivo* (290). After uptake by APCs, PLGA nanoparticles are degraded and their contents released into the endosome. Intracellular degradation of PLGA is more complicated than extracellular degradation as it involves hydrolytic enzymes and catalytic activity of the acidic endosomal/lysosomal compartments, in addition to random hydrolysis of backbone esters (291).

1.7.3 Adjuvant activity of PLGA nanoparticles

PLGA nanoparticles can be used to formulate a wide variety of antigens for targeted delivery to APCs, including DCs. In addition, they can also encapsulate the antigen and adjuvant for delivery to the same cell taking up the particles. Antigens delivered by PLGA particles can be processed and presented by both the MHC class I and MHC class

II pathways. In addition to cellular uptake, antigens released into the extracellular medium from PLGA particles can bind to antibodies on B lymphocytes and generate humoral immune responses.

Protein antigen delivery through PLGA microparticles was initially tested for the induction of humoral immune responses (292-297). In addition, the induction of systemic and mucosal immune responses has been reported with antigens encapsulated in PLGA microparticles (294-296). Furthermore, parenteral immunization with antigens encapsulated in PLGA microparticles generated systemic humoral responses comparable to those elicited by complete freunds adjuvant (CFA) (297) and Alum (279, 292) or even higher than CFA (298).

Induction of humoral immune responses by antigens encapsulated in PLGA microparticles was initially investigated. However, as cancer and other infectious diseases require the generation of cellular immune responses, PLGA microparticles were later investigated to achieve this outcome. Superior T cell proliferative responses were elicited following encapsulation of protein and peptide antigens in PLGA microparticles (292, 294, 295, 298, 299). In one study, the extent of T cell proliferative responses was found to be highest with rapid antigen release microparticles fabricated with the low molecular weight PLGA polymer (298). In addition, the particle size was also found to have a profound impact on the immune responses (298, 300). The superiority of the PLGA microparticle system to generate Th1 immune responses was reported in a study where HIV gp120 encapsulated in PLGA microparticles induced T cells that secreted high levels of IFN-γ and undetectable levels of IL-5 (299). However, the same antigen

when admixed with Alum induced T cells that secreted moderate to high levels of IL-4 and IL-5 and very low levels of IFN-γ, a profile consistent with a Th2 response.

1.8 Research proposal

The goal of this research was to evaluate PLGA nanoparticles as delivery vehicles for targeting antigens to DCs for the induction of cellular immune responses. A therapeutic cancer peptide antigen (a 25 mer human MUC1 lipopeptide) was encapsulated in PLGA nanoparticles and evaluated for its ability to elicit Th1 responses and overcome self-tolerance mechanisms. Furthermore, this study systematically examined the potential of the PLGA nanoparticle system to deliver antigen and immunomodulators to DCs for inducing DC maturation and generation of cellular immune responses *in vitro* and *in vivo*.

1.8.1 Hypotheses

1. The manner in which an antigen is administered dictates the nature of the ensuing immune response.
2. Microencapsulation of antigens will circumvent the need for carrier proteins and bias the immune responses towards a Th1 response.
3. Mycobacterium compounds like Lipid A analogues will enhance Th1 responses and overcome self-tolerance when incorporated with antigens in nanoparticles.
4. PLGA nanoparticles are efficiently internalized by DCs- a required step for the induction of efficient T cell proliferative responses.

1.8.2 Objectives

1) To encapsulate a 25mer human MUC1 lipopeptide in PLGA nanoparticles and characterize the formulation and evaluate it's associated immune responses in wild-type and human MUC1 transgenic mice.
2) To generate cultures of DCs from murine bone marrow progenitors and evaluate their uptake of PLGA nanoparticles.
3) To assess phenotypic and functional maturation of DCs following delivery of MPLA in PLGA nanoparticles.
4) To investigate the ability of DCs loaded *in vitro* with PLGA nanoparticles containing antigen and MPLA to elicit T cell responses *in vitro* and *in vivo* and over come self-tolerance mechanisms.

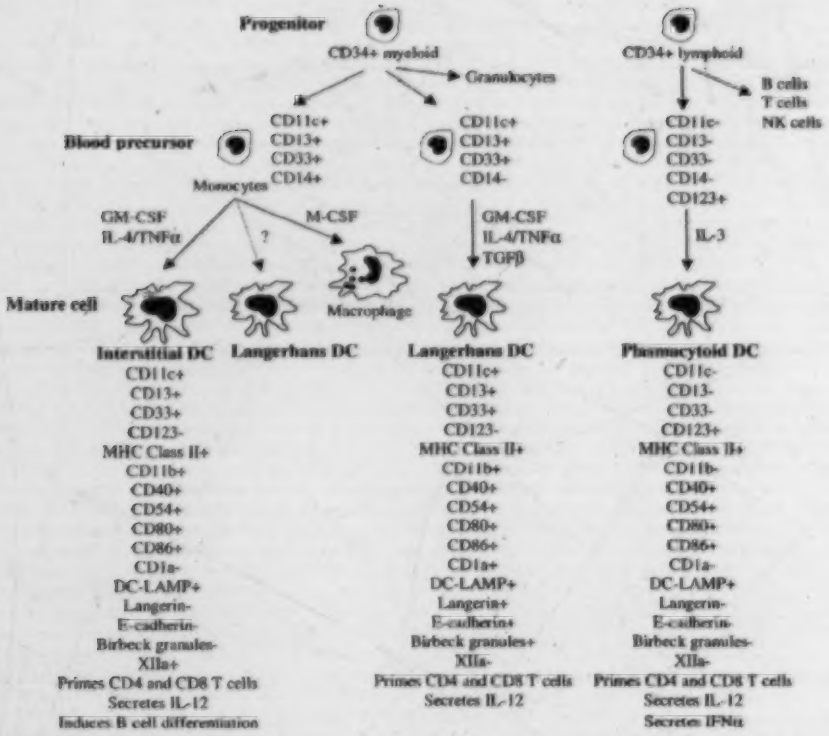

Figure 1-1. Generation of human DCs from $CD34^+$ myeloid and lymphoid progenitor cells (adapted from reference (301)).

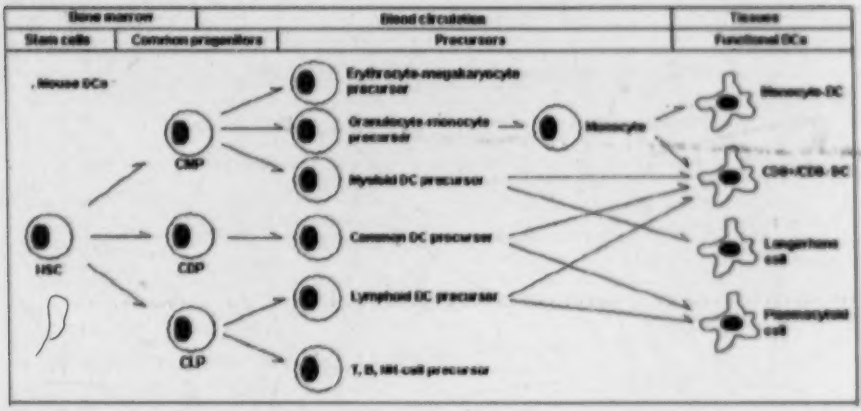

Figure 1-2. Pathways for generation of mouse DCs from hematopoietic stem cells (adapted from reference (302))

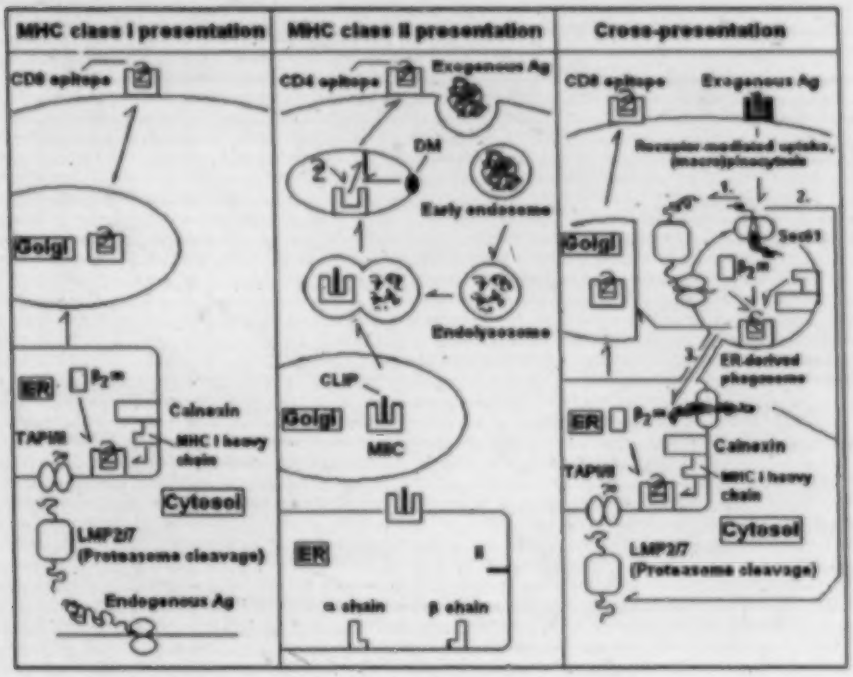

Figure 1-3. MHC class I and MHC class II antigen presentation pathways (adapted from reference (302))

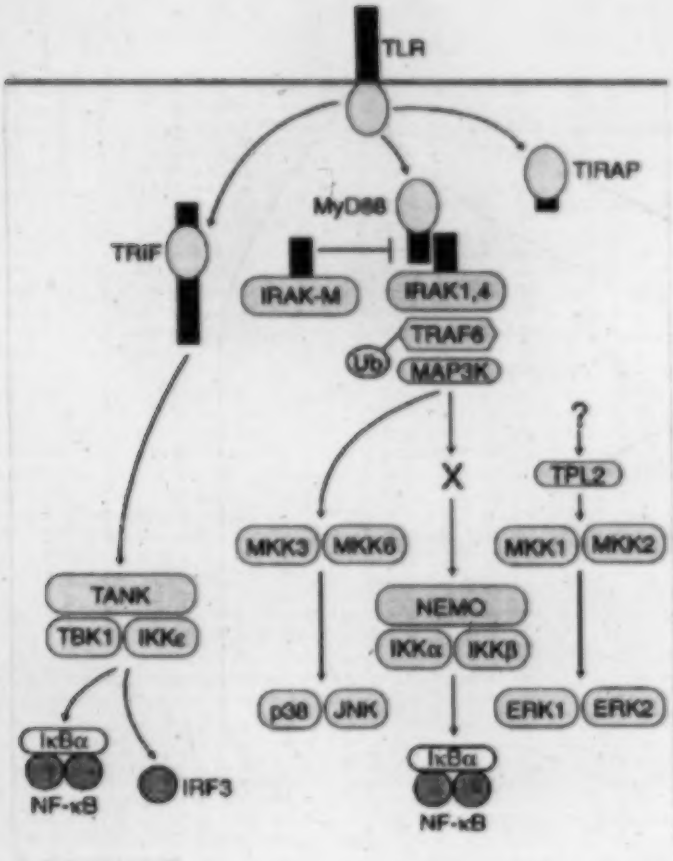

Figure 1-4. TLR signaling pathways (adapted from reference (303))

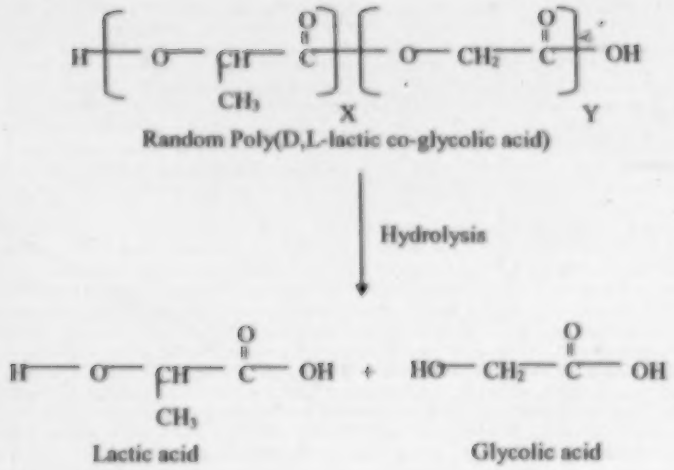

Figure 1-5. Structure and biodegradation products of PLGA

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Chapter Two

Formulation and preclinical evaluation of a cancer vaccine formulation comprising of 25 amino acid MUC1 lipopeptide antigen encapsulated in poly(D,L-lactic-co-glycolic acid) nanoparticles

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2.1 Introduction

In light of the shortcomings of conventional cancer treatments, immunotherapy and the development of cancer vaccines represent attractive prospects for cancer research. MUC1 is a high molecular weight mucin-like type I transmembrane glycoprotein (> 200 k Da) normally expressed on most epithelial cell surfaces and largely comprises of variable number (25-100) tandem repeats of 20 amino acid units (PDTRPAPGSTAPPAHGVSTA) with attached carbohydrate side chain moieties (1). It is normally expressed in a polarized fashion in the luminal ducts of the breast, ovary, pancreas, lung, and colon. Cancer associated MUC1 is over expressed and underglycosylated exposing the normally cryptic peptide sequences (2, 3). The increase in its expression and the loss of polarity on the cell surface correlates with tumour progression and metastases (4-6). MUC1 peptides corresponding to the tandem repeat and cytoplasmic domains are thus important candidates for the design of therapeutic vaccines (7-9) for a number of common cancers including that of breast, prostate, ovary, lungs, heart, and pancreas (10-14). Since majority of the cancer-associated antigens are self-antigens, immune recognition and overcoming self-tolerance is a major challenge in cancer immunotherapy.

The mode of antigen delivery and the 'context' of antigen processing and presentation by DCs profoundly influences the quality and the quantity of immune responses (15, 16). Many strategies have been investigated for the induction of immune responses against MUC1 (17-22). However, a proven delivery system that can generate a clinically effective immune response against MUC1 expressing tumours still remains to be achieved.

Most of the cancer associated antigens when administered alone do not sufficiently generate anti cancer T cell responses. In an attempt to improve the immunogenicity, cancer antigens are being administered with adjuvants, haptens, and carrier proteins. However, not many of these 'immunogenicity helpers' are currently accepted for human use and need thorough validation for clinical applications. 'Alum', one of the FDA approved adjuvant induces humoral immune responses (23) that are not beneficial in treating cancer which needs a robust cell mediated immune response. The recently approved adjuvant MF59, a squalene based oil/water emulsion is yet to be tested for a broad range of anti cancer vaccines (24-26).

Nanoparticles fabricated with poly (D,L-lactic-co-glycolic acid) (PLGA) are 'prototype' non-toxic delivery system that have been investigated for co-delivery of antigens and immunomodulators for the induction of cellular and humoral immune responses (discussed in chapter 1). In this chapter, the pharmaceutical and immunological evaluation of a single emulsion PLGA nanoparticle formulation encapsulating a 25 mer human MUC1 lipopeptide (BLP25) and monophosphoryl lipid A (MPLA), a TLR4 (Toll like receptor) ligand is reported. As human MUC1 is a foreign antigen when administered in wild-type mice, the efficacy of this vaccination strategy for overcoming self tolerance was also tested in human MUC1 transgenic mice (MUC1. Tg mice) where MUC1 is expressed as a self antigen (27). The results shed light on the superiority of the formulation in attaining a high degree of antigen encapsulation efficiency and its ability to generate robust T helper immune responses that can overcome self tolerance *in vivo*.

2.2 Materials and Methods

2.2.1. Mice

Male C57BL/6 mice were purchased from the Jackson laboratories (Bar Harbor, ME) and housed at the University of Alberta's Health Sciences Laboratory Animal Services (HSLAS). Male C57BL/6 mouse strain transgenic for human MUC1 (MUC1.Tg) were kindly provided by Dr. Sandra Gendler (Mayo Clinic, Scottsdale, AZ) and bred at the University of Alberta's transgenic animal breeding facility at the HSLAS. Polymerase chain reaction (PCR) sample amplification from DNA extracted from the ear biopsies using MUC1 primers (bp745-765 and bp1086-1065) (Biomira Inc., Edmonton, AB, Canada), was used to routinely identify MUC1 positive mice in the colony. MUC1 expression in various organs and tissues from selected mice was analyzed by immunohistochemical staining with mAb against MUC1 antigen (27). All animal studies were carried out in compliance with the University of Alberta's guidelines for the care and use of laboratory animals. Unless otherwise stated all animals were 6-10 weeks old at the time of vaccination.

2.2.2 Materials

Human MUC1 lipopeptide- STAPPAHGVTSAPDTRPAPGSTAPP-Lysine (palmitoyl)G, (BLP25); STAPPAHGVTSAPDTRPAPGSTAPP, a 25 amino acid human MUC1 peptide (BP151); a 24 mer human MUC1 peptide TAPPAHGVTSAPDTRPAPGSTAPP (BP1-065); DSTSSPVHSGTSSPATSAPEDSTS, a mouse MUC1 peptide (BP1-020); PKVLHKRTLGLPAMSTTDLEAYFKD, a Hepatitis B virus x antigen peptide (HBVxAg); ISQAVHAAHAEINEAGR, a chicken ovalbumin

peptide (323-339) (BP1-037); ISQAVHAAHAEINEAGRK(Pal)G, a ovalbumin lipopeptide (BP204); PTHASPASTPAAPGPTGASRTPVDPK(Pal)G, a scrambled human MUC1 peptide (BP224); GVTSAPDTRPAPGSTA, a human MUC1 peptide sequence (BP007); and MPLA were kindly provided by Biomira Inc. PLGA co-polymer, monomer ratio 50:50, m.w 7000 Da, was purchased from Birmingham polymers (Birmingham, AL). Polyvinyl alcohol (PVA) (87-89% hydrolyzed, m.w 31-50,000 g/mol) was obtained from Aldrich Chem (Milwaukee, WI). HPLC-grade acetonitrile (ACN), chloroform (CHL), and methanol were obtained from Caledon (Mississauga, ON, Canada). Tetrahydrofuran (THF) was obtained from Fisher Scientific (Fair Lawn, NJ). Spectroscopic grade Trifluoroacetic acid was obtained from Sigma. Murine IFN-γ ELISA kit was purchased from E-Biosciences (San Diego, CA). QCL-1000 Chromogenic Limulus Amebocyte Lysate (LAL) endotoxin detection kit was obtained from BioWhittaker (Walkersville, MD).

2.2.3 Preparation of BLP25 and MPLA nanoparticles

PLGA nanoparticles containing BLP25 with or without MPLA were prepared as oil/water single emulsion formulation by the solvent evaporation method. Briefly, BLP25 solution (50-100 µL, 2.5% (w/v) in 1:4, methanol: chloroform) was added to the PLGA solution in CHL (200 µL, 50% w/v). For the preparation of nanoparticles containing MPLA, 200 µg of MPLA in 100 µL of 1:4 methanol-chloroform mixture was added to the polymer-chloroform solution. Two milliliters of PVA solution [9% w/v PVA in PBS (pH-7.4)] was emulsified with the polymer solution using a microtip sonicator (Heat systems Inc., Farmingdale, NY). The resulting emulsion was then transferred drop-wise

into a beaker containing stirring 8 mL of PVA solution. The emulsion was allowed to evaporate under continuous stirring and nanoparticles were collected after 2.5 h by centrifugation at 17000 × g for 10 min at 4°C. The nanoparticles were washed twice with cold deionized water, lyophilized, and stored at -20°C until further use.

2.2.4 Characterization of nanoparticles

2.2.4.1 Particle size analysis

Nanoparticles were suspended in filtered PBS and the mean hydrodynamic diameter and polydispersity determined by dynamic light scattering methodology using a particle size analyzer (Zetasizer 3000, Malvern, UK).

2.2.4.2 Surface morphology of nanoparticles

The surface morphology of nanoparticles was examined by dispersing PLGA nanoparticles suspension on a metal stub and air drying. The metal stub was then placed in a sputter coater (S150B; BOC Edwards, Sussex, UK) for 40 s to produce a gold coating of approximately 30 nm in thickness. The nanoparticles were then visualized under a scanning electron microscope (Hitachi S-2500, Hitachi, Ltd., Tokyo, Japan).

2.2.4.3 Validation of extraction methods

Spiking studies were performed to demonstrate the applicability of three extraction methods (ACN, THF, and CHL/water extraction) for quantification of BLP25 encapsulated in PLGA nanoparticles. Briefly, 10 mg of empty PLGA nanoparticles were spiked with 50 or 100 μg of BLP25 and freeze-dried overnight. A relatively hydrophilic peptide- HBVx Ag peptide was also spiked onto empty nanoparticles to validate the

extraction methods. The lyophilized samples were subjected to extraction by the three methods and the peptide recovery determined by RP-HPLC.

In the first method (ACN extraction), ACN was added to the freeze dried mixture of peptide and empty nanoparticles and shaken for 30 min. The suspension of dissolved polymer and precipitated peptide and PVA was centrifuged at 7000 × g for 10 min and the supernatant discarded. The pellet was resuspended in PBS and the peptide content estimated following injection of 200 µL of the sample into the RP-HPLC system. In the second method (THF extraction), the freeze dried mixture of peptide and empty nanoparticles was suspended in THF and shaken for 30 min followed by addition of PBS. The suspension containing the precipitated polymer was centrifuged at 7000 × g for 10 min and the pellet discarded. Two hundred microliters of the supernatant was injected into the RP-HPLC system. In the third method (CHL/Water extraction), CHL was added to the freeze dried mixture of peptide and empty nanoparticles and shaken for 15 min. An equal volume of PBS was then added and the tube was allowed to shake for 30 min and centrifuged at 7000 × g for 10 min. The aqueous phase was carefully removed and 200 µL of the sample injected into the RP-HPLC system. All samples were passed through a 0.22 µm filter before injection into the RP-HPLC system.

2.2.4.4 MUC1 lipopeptide stability

To study for any degradation of BLP25 after freeze drying, 500 µL of 30 µg/mL solution of BLP25 was freeze dried and later reconstituted in PBS and analyzed for lipopeptide content by RP-HPLC.

2.2.4.5 Analysis of BLP25 in PLGA nanoparticles

BLP25 encapsulated in PLGA nanoparticles was extracted by the ACN, THF, and CHL/Water extraction methods and quantified by RP-HPLC similar to the protocol mentioned earlier for the validation studies.

2.2.4.6 Reverse phase HPLC conditions

Two hundred microliters of the sample was injected into a Waters HPLC system (Waters, Mississauga, ON, Canada) equipped with a C_{18} reverse phase column (8 × 100 mm, 15 µm particle size, Pore size- 300 A°) and a UV detector (Waters 486) set at 210 nm and a Gilson autoinjector (Mandel Scientific, Guelph, ON, Canada). The mobile phases employed were 10% ACN in water with 0.1% trifluoroacetic acid (Solvent A) and 70% ACN in water with 0.085% trifluoroacetic acid (Solvent B). The column was equilibrated with 75% A and 25% B prior to each run. Samples were eluted on a linear gradient of 25% to 100% B over 12 min at a fixed flow rate of 0.8 mL/min. The amount of peptide in each sample was calculated using a standard curve generated with known concentrations of the peptide. For analysis of HBVx Ag, samples were eluted on a linear gradient of 11 to 40% B over 18 min at a flow rate of 1 mL/min. Data was collected and processed with an AllchromTM data system (Alltech Assosciates Inc., Deerfield, IL). The calibration curve used for BLP25 quantification was linear over the range of 5 to 200 µg with r^2= 0.9986.

2.2.4.7 Quantification of MPLA loading in PLGA nanoparticles

MPLA loading in PLGA nanoparticles was determined as described earlier (28). Briefly, MPLA was derivatized by reacting with a solution of dinitrobenzyloxyamine hydrochloride (Sigma-Aldrich) in anhydrous Pyridine (10 mg/mL; 200 µL per mg MPLA

extracted from nanoparticles). The samples were dried under N_2 gas at 60 °C and then dissolved in 500 µL $CHCl_3$-CH_3OH (2:1, v/v). The HPLC assay was carried out with µBondapack™ C_{18}, 10 µm particle size, 3.9 × 300 nm stainless steel column (Waters corp., Milford, MA). The mobile phases comprised of 5 mM tetrabutylammonium dihydrogeNPhosphate (TBAP) (Sigma) in ACN-water (95:5, v/v) and 5 mM TBAP in isopropanol-water (95:5, v/v). The sample was eluted on a linear gradient of 10 to 80% B over 15 min and restoring to 10% B over 30 min. The samples were detected at 254 nm with an UV-detector. Data was collected and processed with an AllchromTM data system. The calibration curve used for the detection of MPLA was linear over the range of 50 to 250 µg with a correlation coefficient of r^2= 0.9969.

2.2.5 *In vitro* release of BLP25 from PLGA nanoparticles

Five milligrams of BLP25 containing PLGA nanoparticles was suspended in 1 mL of PBS at pH-7.0 in different eppendorf tube and incubated in a water bath at 37°C under gentle shaking. At each designated time point, three tubes were removed and supernatant collected after centrifugation at 7000 × g for 10 min. The BLP25 released into the PBS medium (supernatant) was quantified by RP-HPLC.

2.2.6 Endotoxin test

The presence of any endotoxin in PLGA nanoparticles was determined by the QCL-1000 Chromogenic LAL endotoxin testing kit. Briefly, 1 mg of empty PLGA nanoparticles was suspended in pyrogen free water and shaken vigorously for 1 h at room temperature. The nanoparticle suspension was centrifuged and the supernatant tested

using the LAL kit according to the manufacturer's directions. The nanoparticle formulations were found to be relatively free of any endotoxins (< 0.1 EU/mg of polymer mass).

2.2.7 Immunization of mice

For studies employing wild type mice, C57BL/6 mice (n= 5 each) were immunized twice (i.d., two weeks apart) with one of the three formulations- MPLA nanoparticles [MPLA] NP, BLP25 nanoparticles [BLP25] NP, and BLP25 and MPLA nanoparticles [BLP25 + MPLA] NP. The administered doses of the BLP25 and MPLA were 50 μg and 20 μg respectively per mice. Ten days after the last immunization, mice were euthanized and blood samples collected through cardiac puncture for serum IgG and IgM analysis and inguinal lymph nodes harvested for T cell proliferation and cytokine analysis. For studies using MUC1.Tg mice, mice (n = 7-8 each) were immunized i.d. with [BLP25 + MPLA] NP on days 1 and 14 followed by evaluation of T cell responses on day 28.

2.2.8 Immune responses studies

2.2.8.1 T cell proliferation assay

The specificity of the immune responses generated following immunization with the nanoparticle formulations was determined by antigen specific recall T cell proliferation assay as described earlier (29). Briefly, inguinal lymph nodes of the immunized mice were harvested and single-cell suspensions prepared by smearing cell aggregates between two frosted slides. Nylon wool columns (type 200 L, Robbins Scientific Corp., Sunnyvale, CA) were used to enrich T cells from cell suspension. CD4 and CD8 T cells

were isolated using Cellect T cell isolation columns (Cytovax Biotechnologies Inc., Edmonton, AB, Canada) according to the manufacturer's directions. Purified T cells (5×10^5 cells/well) were incubated with 20 μM of positive or negative recall antigens and irradiated (3000 rads) autologous spleenocytes obtained from unimmunized mice that served as antigen presenting cells (APCs; 1×10^6/well). The co-cultures were conducted in a 96 well plate and incubated in a humidified incubator set at 37 ℃ with 5% CO_2. T cell proliferation was assessed based on the incorporation of [^{3}H]-thymidine added during the last 18 h of a three day co-culture.

2.2.8.2 Cytokine analysis

Parallel T cell cultures were set up under conditions identical to the T cell proliferation experiment and culture supernatants were removed after 72 h and frozen at -20℃. Interferon gamma (IFN-γ) level in the culture supernatant was determined by sandwich ELISA in a 96 well microtiter plate using a microplate reader (Powerwave with KC Junior software; Bio-Tek, Winooski, VT) at optical density (O.D) of 450 nm with reference set at 570 nm according to the manufacturer's directions.

2.2.8.3 Anti-MUC1 antibody analysis

End point titration methods to determine the MUC1 specific IgG and IgM antibodies in the pooled mouse serum samples from immunized mice were performed using ELISA similar to the protocol mentioned earlier (29). Briefly, 96 well flat bottom NUNC microtiter plates were coated with 500 ng/well of BLP25 and incubated overnight at 4℃. The plates were washed and incubated with serial dilutions of serum samples that included test serum from vaccinated mice, normal serum from unimmunized mice and positive control that included serum from C57BL/6 mice immunized with MUC1 peptide

conjugated to keyhole limpet hemocyanin (KLH) at 25°C for 1 hr. The wells were washed thoroughly and peroxidase conjugated goat anti mouse IgG or IgM antibodies or their respective isotype controls were added and incubated for 1 h at 25°C. The wells were washed and incubated with 2,2'-azinobis[3-ethylbenzothiazolinesulfonate] (ABTS) and a 15 min OD reading at 405 nm was taken using a microplate reader.

2.2.9 Statistical Analysis

The results of the immunological assay are expressed as the mean +/- standard deviation (S.D.) of triplicate wells. An unpaired student's 't-test' was used to compare sample means and assess the statistical significance.

2.3 Results

2.3.1 Particle size analysis and BLP25 extraction and loading efficiency

The mean hydrodynamic particle size (Z average, Continuous analysis) of the [BLP25] NP or [BLP25+MPLA] NP was quite similar, 200-500 nm with polydispersity below 0.1 (Fig. 2-1A and B) after primary lyophilization. The nanoparticles had a smooth and spherical morphology as illustrated using scanning electron micrograph picture of a representative nanoparticle preparation (Fig. 2-1C).

In order to account for any loss or chemical modification or structural degradation of BLP25 after spiking which involved lyophilization, BLP25 solution was freeze dried and resuspended in PBS and analyzed by RP-HPLC. Comparison of the amount of BLP25 recovered after freeze drying with that of freshly injected sample of the same concentration allowed for determination of peptide recovery. It also gave information for

any degradation after lyophilization. The recovery of BLP25 after freeze drying was greater than 94 ± 2%. There were also no visual difference between the RP-HPLC chromatograms of lyophilized and the fresh samples of BLP25 that indicated absence of any degradation after freeze-drying. Lyophilization is carried out after the final washing step during the formulation of BLP25 in PLGA nanoparticles. The results indicated that BLP25 maintained its structural integrity after freeze drying which thus proved it worthy for recovery studies that involved spiking, lyophilization, and extraction.

In order to select for an appropriate method for extraction of BLP25, the efficiency of three extraction methods to recover the encapsulated BLP25 from PLGA nanoparticles was analyzed. Empty nanoparticles were spiked with known amounts of BLP25 or HBVx Ag and the mixture subjected to extraction by the three extraction procedures. Finally, extracted peptide was quantified by RP-HPLC. Recovery using THF and ACN extraction methods ranged between 87 and 93% respectively while the CHL/water extraction demonstrated the lowest recovery of 25% (Table. 2-1). CHL/water extraction method proved to be more efficient for recovery of a hydrophilic peptide- HBVx Ag (Table. 2-1). Thus the amphiphilic peptide BLP25 was recovered equally well by the THF and ACN extraction methods in contrast to the more hydrophilic peptide (HBVx Ag) whose recovery was the greatest with the CHL/water extraction process. Thus, selection of the solvent required for extracting the peptide from PLGA nanoparticles was largely influenced by the relative solubility of the peptide antigen.

Once the most efficient methods for extracting the BLP25 were determined and validated, the BLP25 encapsulated inside the PLGA nanoparticles was quantified by these methods. The results demonstrated that using the THF extraction method, the

amount of detectable BLP25 encapsulated in PLGA nanoparticles was slightly higher when compared to the ACN extraction method (however no statistically significant difference was observed) (Table. 2-2). Consistent with the recovery studies, a very low amount of BLP25 was detected when CHL/water extraction method was employed. To account for any loss of BLP25 during the ACN extraction process, the peptide solution obtained was centrifuged and the pellet, if any, reconstituted in ACN. The ACN solution was then centrifuged and the supernatant discarded and the walls of the centrifuge tube washed thoroughly with 0.1N sodium hydroxide and the washings neutralized and analyzed by RP-HPLC. The chromatogram obtained did not show any detectable lipopeptide peak which thus indicated a complete extraction of BLP25 from PLGA nanoparticles. The BLP25 encapsulation efficiency was estimated to be 82.3 ± 7.9%.

2.3.2 BLP25 release from PLGA nanoparticles

The release of BLP25 from PLGA nanoparticles is critical for the applicability of the nanoparticle formulation to serve as a delivery system. Therefore, the liberation of BLP25 from the nanoparticles was assesed over a period of 30 days. As shown in Fig. 2-2, a characteristic biphasic release of BLP25 from PLGA nanoparticles was observed. After the initial burst release phase observed during the first 12-24 h where approximately 10.3 ± 1.4% of the BLP25 was released, a lag period followed where the lipopeptide was slowly released for up to 96 h which was again followed by a gradual increase in release over 48 h followed by stabilization. After 30 days, a total of 16.6 ± 1.6% of the encapsulated BLP25 was liberated into the medium.

2.3.3 Cellular immune responses in wild-type mice following vaccination with [BLP25+MPLA] NP

The vaccination induced MUC1 specific immune responses were determined by T cell proliferation assay in presence of relevant and irrelevant recall antigens (Fig. 2-3). The antigen specific T cell proliferative responses could be detected in T lymphocytes of wild-type mice vaccinated with the vaccine formulations with the most efficient being the [BLP25+MPLA] NP vaccinated group. The proliferation observed with the irrelevant antigen BP1-037 was minimal or almost equal to the background T cell proliferation obtained without the addition of any recall antigens (No Ag group). Immunization with [BLP25] NP alone generated an antigen specific T cell proliferation which was significantly higher than the T cell proliferative responses obtained following immunization with [MPLA] NP. In order to further validate and confirm the MUC1 specific immune responses and assess the vaccination strategy for induction of effector responses, the production of IFN-γ by T lymphocytes was assayed. As shown in Fig. 2-4, IFN-γ production by T cells was significantly higher in mice vaccinated with [BLP25+MPLA] NP than [BLP25] NP with more than a 9 fold increase in the production of IFN-γ in presence of MPLA. Assessment of antibody response was performed based on analysis of mouse sera by indirect ELISA and the predominant secretion of MUC1 specific IgG over IgM was observed (Fig. 2-5).

2.3.4 [BLP25+MPLA] NP immunization stimulates strong $CD4^+$ lymphocyte responses in MUC1.Tg mice

After observing the generation of potent T cell mediated immune responses in wild-type mice following vaccination with [BLP25+MPLA] NP, the efficiency of this vaccine formulation to break the self tolerance mechanism was evaluated in the MUC1. Tg mice. As illustrated in Fig. 2-6, T lymphocytes obtained from the draining lymph nodes of MUC1. Tg mice immunized with [BLP25+MPLA] NP proliferated strongly in the presence of recall MUC1 peptides. Nanoparticle immunization however activated $CD4^+$ lymphocytes, as only $CD4^+$ T cells and not $CD8^+$ T cells responded to MUC1 recall antigens in the T cell proliferation assay (Fig. 2-7). Production of IFN-γ by the whole T cell population and $CD4^+$ T cells but not by $CD8^+$ T cells following stimulation with cognate MUC1 peptides further indicated that [BLP25+MPLA] NP immunization primarily elicits $CD4^+$ T cell responses at a functional level (Fig. 2-8).

2.4 Discussion

MUC1 mucin is expressed by > 90% of common cancers such as adenocarcinomas of breast, ovary, pancreas, and non-small cell carcinomas of the lung (12). Cancer patients without any prior immunotherapy also have ongoing MUC1 specific humoral immune responses (30). MUC1 specific cytotoxic T lymphocytes (CTLs) have been isolated from tumour draining lymph nodes of patients suffering from breast and ovarian cancers (31). However, these existing immune responses are inadequate and do not translate into a therapeutic benefit as tumours devise a wide variety of mechanisms to inhibit the development of type 1 responses (32) and furthermore delay the induction of effector

CTL responses (33). Reduced $CD4^+$ T helper cell function has also been implicated for the inability of patients to generate a robust immune response and overcome the disease condition (34). BLP25 was selected as the tumour antigen due to its significance as a potential cancer vaccine candidate in the development of therapeutic vaccines for immunotherapy of $MUC1^+$ tumours (35, 36). BLP25, in addition to containing HLA class I binding epitopes also possesses class II binding epitopes (37, 38) that can aid in the induction of $CD4^+$ T cell helper functions.

The formulation of a 24 mer human MUC1 mucin peptide (BP24) encapsulated in PLGA nanoparticles along with MPLA as a potential cancer vaccine formulation was previously reported (29). This nanoparticle formulation generated a modest Th1-promoting immune response that is pivotal for cancer immunotherapy. However, despite the encouraging results obtained with these nanoparticles, the low encapsulation efficiency of the MUC1 peptide due to its diffusion from the internal to the external aqueous phase during the solvent evaporation step of nanoparticle preparation was a potential hurdle for future pharmaceutical acceptance. BLP25 is a 25 mer human MUC1 lipopeptide. Palmitoylation gives the peptide a relatively hydrophobic C-terminal end permitting it to be incorporated into the organic phase thereby allowing the feasibility to make oil in water single emulsion nanoparticles with high encapsulation efficiency. This vaccine formulation is also expected to be superior in generating Th1-type responses as palmitoylation enhances the immunogenicity of peptide antigens (39) and several lipopeptide antigens have been reported to induce CTL responses (40-42).

A systematic characterization of PLGA nanoparticles encapsulating a 25 mer human MUC1 lipopeptide and MPLA has been described in the current study. As the size of the

nanoparticle plays a key role in the type of APCs involved in their uptake (43) and the final outcome of the immune response (44), nanoparticles encapsulating BLP25 were prepared with a mean hydrodynamic diameter of less than 500 nm to support the development of Th1-type immune responses. The burst release of BLP25 from the single emulsion PLGA nanoparticles was found to be 10%. Three extraction methods were tested and validated for extracting the lipopeptide antigen from PLGA nanoparticles by spiking known amount of BLP25 on to empty nanoparticles and extracting out the lipopeptide for subsequent quantification by RP-HPLC. In the CHL/Water extraction method (29), the PLGA co-polymer due to its solubility partitions into the organic phase (CHL). The hydrophilic peptide partitions more favorably into the aqueous phase which is subsequently used for analysis. In the ACN extraction method (45), the polymer and not the peptide solublizes in ACN. The insoluble peptide can then be resuspended in PBS and quantified. In the THF extraction method (46), both the polymer and the peptide are soluble in THF. However, addition of PBS precipitates out the polymer leaving the peptide in the supernatant. The efficiency of BLP25 extraction was found to be the highest (> 90%) when ACN and THF extraction methods were employed. CHL/Water extraction was found to be very inefficient in lipopeptide recovery. One possible reason might be that the BLP25 was associating itself at the interphase due to its aqueous solubility being mediated through the hydrophilic peptide sequence and the hydrophobicity due to the palmitoyl group and thus gets incompletely partitioned into the aqueous medium which was ultimately used for analysis. An encapsulation efficiency of greater than 80% of the BLP25 was attained in the PLGA nanoparticles as determined by both the ACN and THF extraction methods. These results confirmed that the 25 mer

MUC1 lipopeptide sequence can be successfully encapsulated in single emulsion PLGA nanoparticles with a high encapsulation efficiency.

Generation of cellular immune response requires the antigen be taken up by DCs, the most potent APC (47). Previous findings indicated that DCs are key APC's involved in the uptake of PLGA nanoparticles when administered *in vivo* (48). Encapsulation of BLP25 in PLGA nanoparticles would thus deliver BLP25 most efficiently to DCs. MPLA, a non-toxic derivative of lipid A is a TLR4 ligand and is an alternative to highly toxic lipopolysaccharide (LPS) for immune modulation. It is being currently employed in clinical trials as a co-adjuvant for cancer vaccines (49). In order to restrict the exposure of soluble TLR ligands to the systemic circulation, MPLA was encapsulated along with BLP25 in PLGA nanoparticles to provide the TLR mediated danger signal. These antigen containing nanoparticles were hypothesized to deliver the MUC1 antigen and the danger signal to the same DCs which can then further powerfully activate MUC1 specific T cells. The lack of detectable antigen specific T cell immune responses after immunization with MPLA containing nanoparticles confirmed the relative safety of this delivery system. Immunization with BLP25 encapsulated in PLGA nanoparticles generated T cell responses within the lymph node T cell population with T cell stimulation indices greater than 10. A 2 fold increase in MUC1 specific T cell proliferation was observed following co-delivery of MPLA along with BLP25 when compared to [BLP25] NP vaccination. The enhanced T cell responses generated following vaccination with [BLP25+MPLA] NP indicated that the DCs had efficiently taken up the nanoparticles and processed and presented the encapsulated antigen in a superior fashion to T cells. The entrapped MPLA was furthermore activating the DCs through its immunomodulatory properties and thus

contributed to the observed enhancement in T proliferative responses. The secretion of high levels of IFN-γ by T cells of mice immunized with [BLP25+MPLA] NP in the current study indicated the predominant induction of Th1 promoting immune responses by this mode of vaccine delivery.

The MUC1 transgene in the mouse model, driven by its endogenous promoter is expressed in a pattern and level similar to that observed in humans. These transgenic mice express human MUC1 as a self antigen and have the high affinity T cells recognizing MUC1 deleted during T cell ontogeny. These transgenic mice are tolerant to stimulation by MUC1 antigen making them an ideal preclinical model to evaluate immunotherapies designed to overcome tolerance as in the case of cancer antigens (27). Immunization with irradiated MUC1-positive tumour cells (50) or adoptive transfer of immune cells from MUC1.Tg mice primed *in vitro* with B16.MUC1 tumour cells to wild type C57BL/6 mice (51), and passive transfer of anti-MUC1 antibodies (52) have all failed to overcome tolerance to MUC1. Immunization with fusions of DCs and tumour cells could successfully break the tolerance in MUC1.Tg mice (8, 21, 50, 53-55). The current findings indicated that delivery of BLP25 and MPLA in PLGA nanoparticles can overcome self-tolerance mechanisms and induce efficient T cell proliferative responses in MUC1 Tg. mice. However, the lack of detectable MUC1 specific proliferation of $CD8^+$ T cells of immunized MUC1. Tg mice was quite intriguing. Literature suggests that the immune responses against MUC1 in both MUC1-positive tumour bearing transgenic mice as well as cancer patients are known to generate low frequency of CTL's and a low titer of antibodies of IgM isotype with apparently no class switching (34, 56).

The results of this study provide useful information on the immunogenicity of the PLGA nanoparticle formulation containing BLP25 and MPLA. One major limitation of this study is that, $CD8^+$ T cell responses could not be observed following vaccination of MUC1.Tg mice with [BLP25+MPLA] NP. Our preliminary results indicated vaccination of MUC1.Tg mice with [BLP25+MPLA] NP does not generate any detectable CTL responses. However, the same vaccine formulation protected the mice against tumour cell challenge with $MUC1^+$ tumour cells. Thus, it is very much likely that $CD4^+$ T cells play a crucial role in controlling tumour progression.

The major goal of the current study was to develop a cancer vaccine formulation using the epitopes of MUC1 antigen encapsulated in biodegradable polymeric nanoparticles for clinical applications in tumour immunotherapy. The current findings indicated that BLP25 and MPLA could be successfully encapsulated in PLGA nanoparticles with a high encapsulation efficiency and these nanoparticles have the capability to induce Th1-type immune responses. Furthermore, this vaccine formulation is capable of overcoming self-tolerance mechanisms. In summary, the results of this study are very encouraging and foretell the promising future of BLP25 and MPLA containing PLGA nanoparticles in the field of cancer vaccines.

Table 2-1

Extraction efficiency of THF, ACN, CHL/Water methods for quantification of BLP25 spiked onto empty PLGA nanoparticles

Peptide	Amount spiked	Extraction efficiency ± S.D.		
		THF	ACN	CHL/Water
BLP25	50 µg	93.9 ± 5.4%	85.2 ± 0.17%	23.5 ± 0.66%
	100 µg	93.5 ± 1.08%	91.2 ± 1.53%	30.7 ± 3.3%
HBVx Ag	100 µg	14.74 ± 0.4%	62.3 ± 1.3%	85.4 ± 0.8%

Empty PLGA nanoparticles were spiked with known concentrations of BLP25 and HBVx Ag and lyophilized. Peptide from the lyophilized samples was extracted by the three extraction methods and quantified by RP-HPLC as described in the *Materials and Methods* section to test the extraction efficiency. Extraction efficiencies are represented as mean ± S.D. between the average means of three independent samples. Unspiked PLGA nanoparticles served as the negative controls.

Table 2-2

Quantification of loading of BLP25 encapsulated in PLGA nanoparticles as determined by the three extraction methods

Extraction method	Peptide encapsulated	Nanoparticle mass	Peptide conc per milligram ± S.D.
THF	BLP25	5 mg	3.54 ± 0.85 µg
		15 mg	3.49 ± 0.32 µg
ACN	BLP25	5 mg	2.98 ± 0.92 µg
		15 mg	3.52 ± 0.31 µg
CHL/Water	BLP25	5 mg	0.85 ± 0.31 µg
		15 mg	0.84 ± 0.25 µg

Single emulsion PLGA nanoparticles encapsulating BLP25 were subjected to the three extraction methods for quantification of BLP25 loading by RP-HPLC as described in the *Materials and Methods* section. Loading was estimated using two concentrations of the BLP25 nanoparticles (5 and 15 mg). Data are represented as mean ± S.D. between the average means of three independent samples. Empty PLGA nanoparticles served as the negative controls.

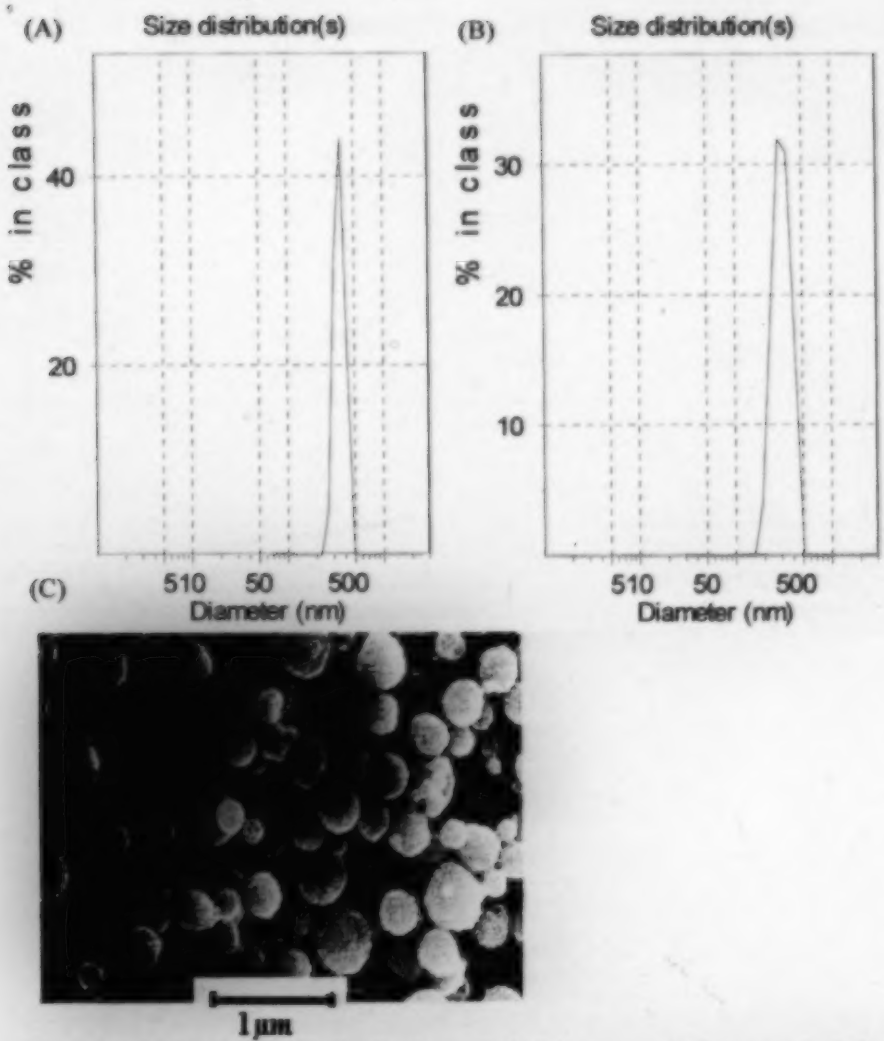

Figure 2-1. Particle size distribution of PLGA nanoparticles encapsulating BLP25 (A) and BLP25+MPLA (B). A representative scanning electron micrograph picture of the nanoparticles depicting the morphological characteristics is shown in (C).

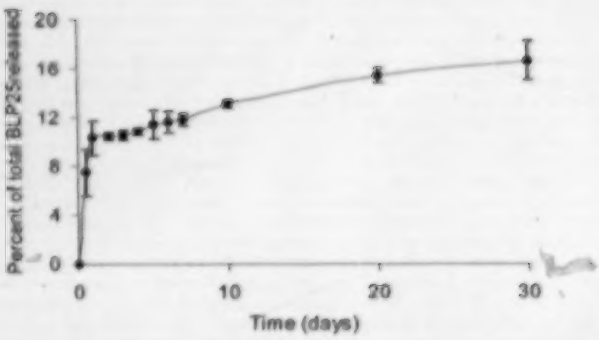

Figure 2-2. *In vitro* release of BLP25 from PLGA nanoparticles. BLP25 nanoparticles were incubated in PBS at 37°C under gentle shaking. At designated time intervals, the samples were centrifuged and the supernatant removed for quantification of BLP25 released into the medium. Error bars represent S.D. between the average means of three independent samples analyzed in triplicates.

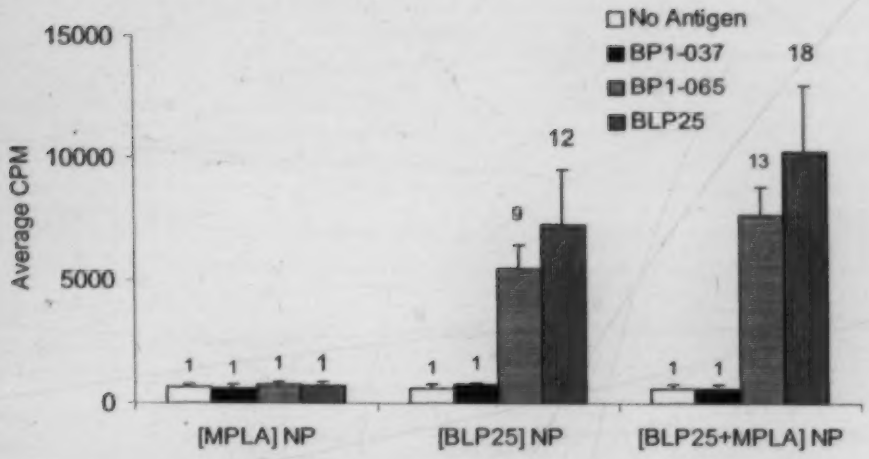

Figure 2-3. Analysis of T cell proliferation assay of T cells isolated from wild-type mice (n=5) immunized with [MPLA] NP or [BLP25] NP or [BLP25+MPLA] NP. C57BL/6 mice were immunized with the nanoparticle formulations. The antigen-specificity of the response was determined by comparing the levels of proliferation (shown as ^{3}H-thymidine incorporation in CPM) after incubation with relevant antigens (BP1-065 or BLP25) to that obtained with irrelevant antigens (BP1-037). The proliferative responses at 2×10^{5} T cells in presence of recall antigens was measured. Error bars represent S.D. between three wells. Unspiked PLGA nanoparticles served as the negative controls. The number above the bar represents the stimulation index (SI) which was calculated as the ratio between the CPM obtained in presence of recall antigen to the background CPM obtained without any antigen. Following [BLP25] NP vaccination, $P<0.001$ between BP1-037 and BP1-065 restimulation and $P<0.01$ between BP1-037 and BLP25 restimulation. Following [BLP25+MPLA] NP vaccination, $P<0.0005$ between BP1-037 and BP1-065 restimulation and $P<0.005$ between BP1-037 and BLP25 restimulation. A representative of two independent experiments is shown.

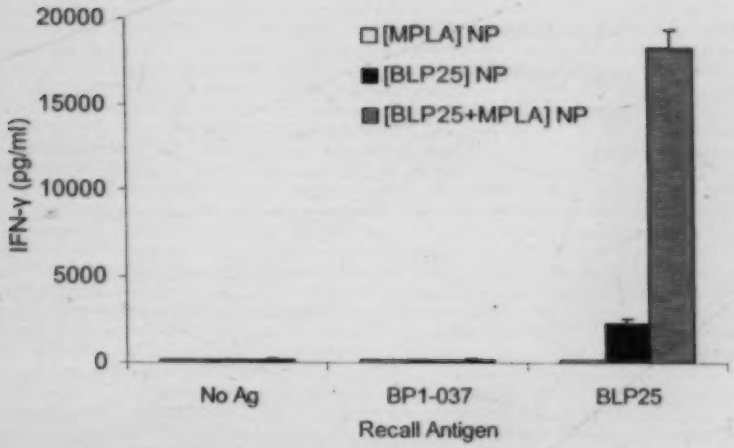

Figure 2-4. Secretion of IFN-γ by T cells isolated from mice (n=5) vaccinated with [MPLA] NP, [BLP25] NP, or [BLP25+MPLA] NP. The cytokine secretion was evaluated in the presence of BLP25 (relevant antigen) or BP1-037 (irrelevant antigen) or no antigen. All samples were performed in triplicates and the error bars represent the S.D between the three wells. $P < 0.5$ between BLP25 and BP1-037 restimulation; following [BLP25] NP vaccination. $P < 0.0005$ between BP1-037 and BLP25 restimulation following [BLP25+MPLA] NP vaccination. $P < 0.00001$ for IFN-γ secretion between [BLP25] NP and [BLP25+MPLA] NP vaccination following restimulation with BLP25. A representative of two independent experiments is shown.

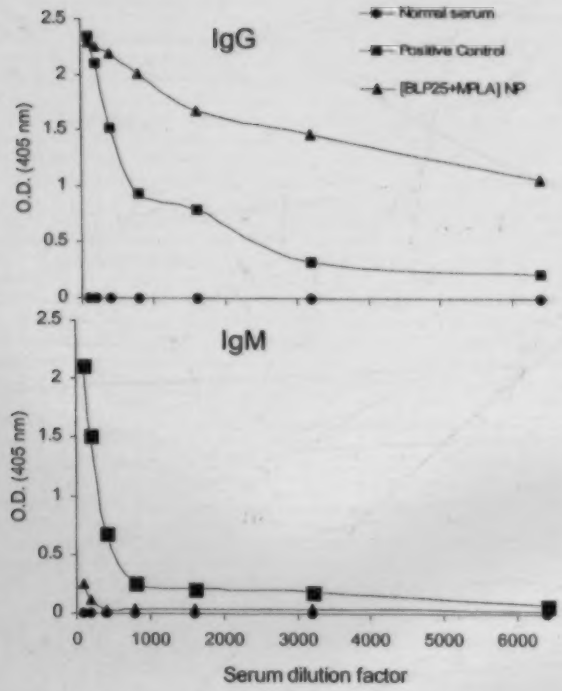

Figure 2-5. Anti MUC1 IgG (A) and IgM (B) responses in C57BL/6 mice (n=5) vaccinated with [BLP25+MPLA] NP. Normal serum was obtained from unimmunized mice and positive control serum was obtained from mice immunized with MUC1 16 mer peptide conjugated to keyhole lymphet hemocyanin (KLH). The O.D. at 405 nm represents the serum antibodies reactive against MUC1 (with background subtracted). The samples were analyzed in triplicates and the data are represented as the average absorbance of three wells. A representative of two independent experiments is shown

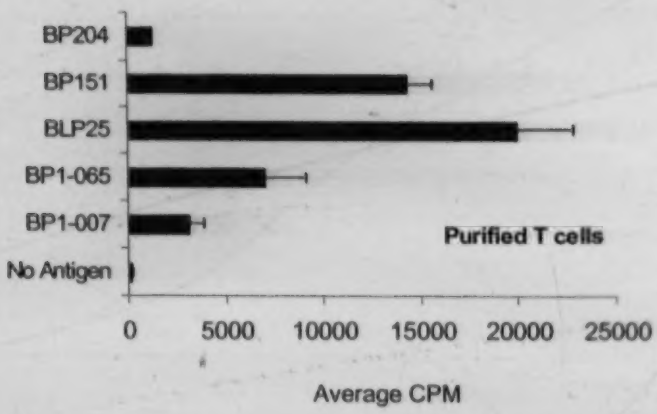

Figure 2-6. Analysis of T cell proliferation assay of T cells isolated from MUC1.Tg mice immunized with [BLP25+MPLA] NP. Human MUC1.Tg mice (n=7/8) were immunized with (BLP25+MPLA] nanoparticles. The antigen-specificity of the response was determined by comparing the levels of proliferation (shown as ^{3}H-thymidine incorporation in CPM) after incubation with relevant antigens (BP151, BP1-065 or BLP25) to that obtained with irrelevant antigens (BP1-007 or BP204). The proliferative responses at 2×10^{5} T cells in presence of recall antigens was measured. All samples were performed in triplicates and the error bars represent S.D. between triplicate wells. $P<0.0005$ for T cell proliferation following restimulation between BP204 and BLP25. A representative of two independent experiments is shown.

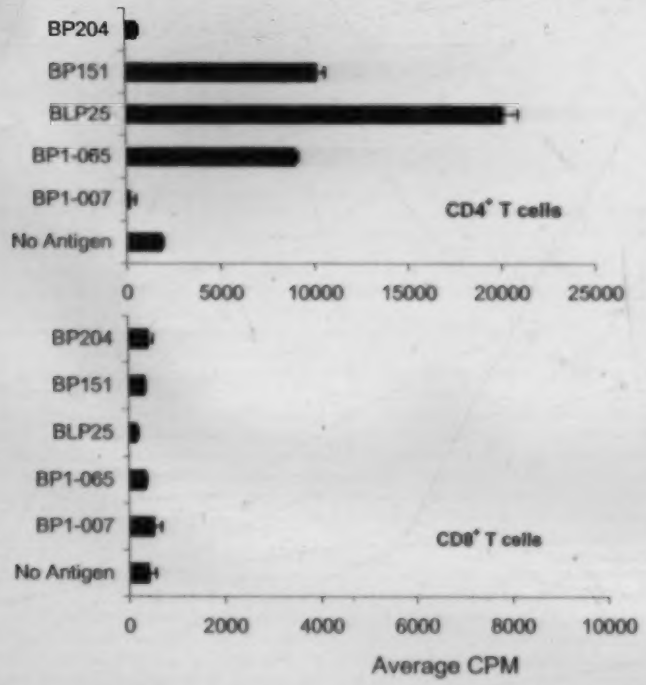

Figure 2-7. Analysis of T cell proliferation assay of CD4 and CD8 T cells isolated from MUC1.Tg mice immunized with [BLP25+MPLA] NP. Human MUC1.Tg mice (n=7/8) were immunized with (BLP25+MPLA] nanoparticles and the CD4 and CD8 T cells isolated by affinity columns through positive selection. The antigen-specificity of the response was determined by comparing the levels of T cell proliferation (shown as ^{3}H-thymidine incorporation in CPM) after incubation with relevant antigens (BP151, BP1-065 or BLP25) to that obtained with irrelevant antigens (BP1-007 or BP204). The proliferative responses at 2×10^{5} T cells in presence of recall antigens was measured. Error bars represent S.D. between the wells in triplicates. $P<0.0005$ for $CD4^{+}$ T cell proliferation following restimulation between BP204 and BLP25. A representative of two independent experiments is shown.

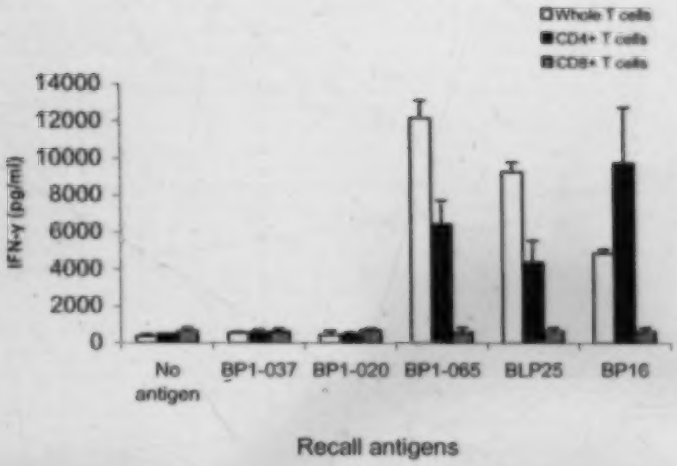

Figure 2-8. Secretion of IFN-γ by whole T cells and CD4 and CD8 T cells isolated from isolated from MUC1.Tg mice immunized with [BLP25+MPLA] NP. Human MUC1.Tg mice (n=7/8) were immunized with (BLP25+MPLA] nanoparticles. Whole T cells were purified by nylon wool columns and CD4 and CD8 T cells isolated by affinity columns through positive selection. The cytokine secretion was evaluated in presence of relevant (BP1-065, BLP25, BP16) or irrelevant (BP1-037, BP1-020) recall antigens. All samples were performed in triplicates and the error bars represent the standard deviation between samples in triplicates. A representative of two independent experiments is shown.

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Chapter Three

Analysis of Poly (D,L-lactic-co-glycolic acid) nanoparticle uptake by murine bone marrow derived dendritic cells *in vitro*

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3.1 Introduction

Dendritic cells (DCs) are the most potent antigen presenting cells (APCs) capable of activating naïve T cells and initiating antigen-specific immune responses against pathogens and foreign antigens (1, 2). A variety of novel delivery approaches to introduce antigens into DCs are being explored for application in tumour immunotherapy (discussed in chapter 1). In this chapter, the applicability of the poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticulate delivery system for introducing antigens into DCs *in vitro* was investigated.

Fluorescent PLGA nanoparticles were prepared and their uptake by DCs *in vitro* evaluated through flow cytometry (FACS) and confocal laser scanning microscopy (CLSM). The phenotype of DC primary cultures internalizing the nanoparticles was assessed by multicolored FACS. The influence of experimental conditions affecting the extent of nanoparticle uptake by DCs and the possible mechanism of nanoparticle uptake investigated. Furthermore, the uptake of particulate antigens over soluble antigens by DCs were compared.

3.2 Materials and Methods

3.2.1 Preparation of fluorescent and ^{125}Iodine-Bovine serum albumin (^{125}I-BSA) containing nanoparticles

PLGA nanoparticles containing tetramethylrhodamine-dextran (TMR-dextran) probe were prepared as a double emulsion formulation by the solvent evaporation technique similar to the protocol described in Chapter 2. Briefly, TMR-dextran (100 µL, 1% w/v solution in PBS (pH-7.4); m.w., 40 kDa; Molecular Probes, Eugene, USA) was

emulsified with PLGA co-polymer (monomer ratio, 50:50; m.w., 50 kDa Birmingham polymers, Birmingham, AL) solution in chloroform (200 µL, 50% w/v) using a microtip sonicator (Heat Systems Inc, Farmingdale, NY) set at level 4 for 15 seconds. The resulting primary emulsion (w/o type) was further emulsified in polyvinyl alcohol (PVA, m.w., 31 to 50 kDa; Aldrich Chemicals, Milwaukee, WI) solution in PBS (2 mL, 9% w/v) to form a secondary emulsion (w/o/w type). The latter was transferred drop-wise into PVA solution (8 mL) and kept under constant stirring for 3 h. After complete solvent evaporation, nanoparticles were obtained by centrifugation at 17,000 × g, washed three times with cold deionized water and freeze dried. Nanoparticles were stored at –20°C until further use. For formulation of ^{125}I-BSA in PLGA nanoparticles, BSA was first conjugated to ^{125}I by the iodogen method similar to a protocol described earlier (3). Briefly, 5 µL of IODO-GEN® (Pierce Biotechnology Inc., Rockford, IL) in chloroform (0.005% v/v) was plated in a glass test tube. Twenty microliters of ^{125}I-NaI (specific activity: 100 mCi/mL; Nycomed Amersham Canada Ltd, Oakville, ON, Canada) and 10 µl of BSA (100 mg/mL) were added in sequence. The reaction was allowed to proceed for 45 min at room temperature. The radiolabelled BSA was separated from free ^{125}I-NaI by Sephadex® G-25M size exclusion columns (Pharmacia Corp, Peapack, NJ). The radioactivity was measured in a 1480 Wizard 3 automatic gamma counter (Wallac, Turku, Finland). The protein fraction used in the study contained 98% of the radio-iodine in a protein bound form as determined by trichloroacetic acid (TCA) precipitation. The conjugated protein was then formulated in PLGA nanoparticles similar to the protocol described for encapsulating TMR-dextran in PLGA nanoparticles.

3.2.2 Characterization of PLGA nanoparticles

PLGA nanoparticles were analyzed for particle size by dynamic light scattering technique using a Zetasizer 3000 (Malvern, UK). The quantification of TMR-dextran in nanoparticles was done as described earlier (4). Briefly, 2 mg of TMR-dextran containing PLGA nanoparticles was stirred in 1 mL of 5% sodium dodecyl sulfate/0.5 M sodium hydroxide overnight. The hydrolyzed samples were centrifuged and the supernatant containing the fluorescent probe collected and neutralized to pH 7 with 1 M HCl. The samples were transferred to a 96 well plate and the absorbance measured at 555 nm by a scanning spectrophotometer (Powerwave X; Bio-Tek Instruments Inc, Winooski, VT). Data analysis was performed by KC Junior software. A standard curve with known concentrations of TMR-dextran was obtained and the percent loading of TMR-dextran was calculated by comparing the UV absorbance of the sample from the calibration curve.

3.2.3 Culture of murine bone marrow derived DCs

Bone marrow derived DCs were generated from C57BL/6 mice (bred in Health Sciences Laboratory Animal Services, University of Alberta, Edmonton, Canada) as described earlier (5). Briefly, bone marrow cells from femurs of 8-12 week old female mice were cultured in 100 mm bacteriological grade petri-dishes (Fisher Scientific, NJ) in complete RPMI [cRPMI; RPMI-1640 containing 10% Fetal bovine serum (FBS, Invitrogen, Grand Island, NY), 100 U/mL Penicillin and 100 µg/mL Streptomycin (Invitrogen), 50 µM 2-ME (Invitrogen), and 2 mM L-glutamine (Invitrogen) in the presence of recombinant murine granulocyte macrophage colony stimulating factor

(rmGM-CSF; 20 ng/mL; Peprotech, Rockville, MA)]. On day 3, the cultures were supplemented with rmGM-CSF in cRPMI followed by half-feeding on day 6. Day 7 DC cultures were used for further studies. DCs were characterized with respect to cell surface expression of CD11c, CD86, and MHC Class II molecules. The purity of DCs on day 8 of the primary culture was greater than 70% ($CD11c^+$).

3.2.4 Uptake of PLGA nanoparticles by DCs

3.2.4.1 Characterization of PLGA nanoparticle uptake by flow cytometry

For characterization of PLGA nanoparticle uptake by DCs, TMR-dextran containing PLGA nanoparticles were incubated with day 7 DC primary cultures (100 μg/mL in 100 mm bacteriological grade petri-dishes) for 24 h. The non-adherent and semi-adherent cells were harvested and stained with titrated amounts of CD11c, MHC class II, and CD86 mAbs in cold FACS buffer (PBS containing 5% FCS and 0.05% sodium azide). Staining was also performed with isotype control antibodies to set the threshold for the fluorescence. The threshold was set such that 98% of the isotype control mAb stained cells fell below 10^1 on the log scale measuring the florescence intensity (X-axis). To determine the optimal stage of DCs that favorably take up PLGA nanoparticles, 6 to 9 day old DC primary cultures were co-incubated with TMR-dextran containing PLGA nanoparticles for 24 h. The cells were harvested and analyzed by FACS. In another experiment, the amount of fluorescent PLGA nanoparticles added to each DC culture dish was varied from 25 to 400 μg/mL (0.5 mg to 8 mg of nanoparticles in 20 mL of media) and the uptake determined after a 24 h incubation period. The influence of incubation time on particle uptake was determined by co-culturing day 7 DC primary

cultures with 100 μg/mL of nanoparticles and studying their uptake over a 24 h period. All samples were acquired on a Becton-Dickinson FACSort flow cytometer (Franklin Lakes, NJ) equipped with FITC (530/30 nm) and phycoerythyrin (585/42 nm) band pass filters, and data was analyzed with CELLQuest software (Becton Dickinson). All mAbs were purchased from BD Pharmingen, Missisuaga, ON, Canada.

3.2.4.2 Confocal laser scanning microscopy

Day 7 DC primary cultures were transferred into Lab-Tek II eight well chamber slides Nalgene Nunc Int., IL) at 2×10^5 cells/well in 500 μL in cRPMI) and incubated at 37°C for 6 h. Five microliters of TMR-dextran containing PLGA nanoparticles (100 μg/mL) was then added to the wells and continued to incubate for an additional 24 h. Control groups were pre-treated with Cytochalasin B (5 μg/mL; Sigma-Aldrich, ON, Canada) for 30 min, prior to the addition of TMR-dextran containing nanoparticles. After 24 h, cells were washed with cold PBS and stained with the CD11c mAb and the appropriate FITC conjugated second antibody. In some experiments, cells were incubated for 2 min with Alexa Fluor® 488 (Molecular Probes, Eugene, USA) for cell membrane staining. The cells were finally fixed with 4% paraformaldehyde and mounted using a solution of 1,4-Diazabicyclo[2.2.2]octane (2.5% w/v; DABCO, Aldrich chemical Company, WI) and gelatin (7.5% w/v) in 50:50 PBS and glycerol. The CLSM images were taken with a Zeiss 510 NLO Confocal laser scanning microscope (Carl Zeiss Microscope Systems, Jena, Germany). All scans were performed in a multi track mode using a 40 mW Argon laser (488 nm) and a 1 mW Helium Neon laser (543 nm).

3.2.4.3 Competitive inhibition studies to elucidate mechanism of PLGA nanoparticle uptake by DCs

To determine the possible mechanism of PLGA nanoparticle uptake, day 7 DC primary cultures were treated with either Cytochalasin B (5 μg/mL) or mannose (2 mg/mL; Sigma-Aldrich) or dextran (1 mg/mL; m.w., 400 kDa; Sigma-Aldrich) for 30 min prior to the addition of 100 μg/mL of TMR-dextran containing PLGA nanoparticles. Control groups were treated with HBSS. After 24 h, cells were harvested and analyzed by FACS.

3.2.5 Estimation of antigen dose taken up by DCs through delivery in PLGA nanoparticles

To quantify the total dose of antigen taken up by DCs, 100 μg/mL of ^{125}I-BSA containing PLGA nanoparticles (having a total activity of approximately 50,000 cpm) was added to day 7 DC primary cultures. In parallel experiments, DC cultures were incubated with soluble ^{125}I-BSA (added in amounts corresponding to 50,000 cpm activity or higher). After 24 h, the semi-adherent and non-adherent cells were harvested from the culture plate and pure DC populations isolated by the murine CD11c isolation kit (Stem Cell Technologies, Toronto, Ontario) by a protocol described by the manufacturer. The radioactivity incorporated in the isolated DC populations was determined by a gamma counter.

3.2.6 Statistical analysis

The results are expressed as the mean +/- standard deviation (S.D.) of samples in triplicates. An unpaired student's 't-test' was used to compare sample means and assess the statistical significance. P values < 0.05 were considered significant.

3.3 Results

3.3.1 Characterization of PLGA nanoparticles

TMR-dextran containing PLGA nanoparticles had a Guassian distribution (200 – 650 nm) with a mean hydrodynamic diameter of 357 nm and polydispersity below 0.15. Loading of TMR-dextran inside the nanoparticles was 0.5% w/w. The mean particle diameter of the ^{125}I-BSA containing PLGA nanoparticles was 470 nm with a polydispersity below 0.1.

3.3.2 PLGA nanoparticles are efficiently internalized by DCs

To assess the uptake of PLGA nanoparticles by DCs, fluorescent PLGA nanoparticles were incubated with day 7 DC primary cultures for 24 h. The semi-adherent and non-adherent cells were then stained with different surface markers and analyzed by FACS. An increase in cellular granularity (side scatter: SSC) in the population that had taken up the nanoparticles was observed (Fig. 3-1A). Greater than 95% of these high granularity cells were positive for the TMR-dextran signal emitting from the nanoparticles. More than seventy percent of these high granularity cells were CD11c^{+} (Fig. 3-1B), which implied that DCs were the major cell type internalizing the PLGA nanoparticles. The DCs were also in their immature state at the time of phagocytosis due to their superior

uptake capability. The cells taking up the PLGA nanoparticles were also MHC class II and CD86 positive (Fig. 3-1B).

3.3.3 Culture conditions affecting nanoparticle uptake by DCs

Figure 3-2A demonstrates that the percent of DCs internalizing the PLGA nanoparticles slightly increased from day 6 through day 7 and plateaued off by day 8. By day 9, DC cultures showed a decline in their particle uptake ability. The uptake efficiency of DCs was also dependent on the concentration of nanoparticles added to the culture (Fig. 3-2B). The percent of DCs taking up TMR-dextran nanoparticles increased from 58 ± 2 % to 75 ± 2 % when the amount of nanoparticles in the DC culture was increased from 25 to 100 μg/mL (0.5 to 2 mg/20 mL). Whereas, no enhancement in the extent of nanoparticle uptake was observed when the dose of the nanoparticles was increased to 400 μg/mL (8 mg/20 mL), the number of particles present per DC concomitantly increased as observed by an increase in the mean fluorescence intensity (MFI) of the TMR probe. Increasing the nanoparticle concentration from 25 to 400 μg/mL increased the MFI of the probe from 20 ± 5 to 62 ± 2. In a separate experiment, the influence of incubation time on nanoparticle uptake was studied. An optimal uptake of nanoparticles was observed by 24 h of incubation (Fig. 3-2C). For subsequent uptake studies, 50 to 100 μg/mL of PLGA nanoparticles was used to treat day 7 DC cultures and incubated for 12-24 h.

3.3.4 Intracellular localization of PLGA nanoparticles after uptake by DCs

The association of PLGA nanoparticle with DCs was further characterized by CLSM in order to verify whether the nanoparticles were indeed taken up and localized intracellularly within DCs or were simply adsorbed onto the cell surface. Chamber slide DC primary culture preparations exhibited typical DC morphology with the characteristic long dendrites visualized by staining with FITC labelled anti-CD11c mAb (Fig. 3-3A, 3-3B) or Alexa Fluor® 488 (Fig. 3-4). Uptake of TMR-dextran containing PLGA nanoparticles was observed as a non-diffuse red signal inside the cells (Fig. 3-3B). The DC cultures pre-treated with a cytoskeletal inhibitor- Cytochalasin B, prior to the addition of fluorescent PLGA nanoparticles remained TMR-dextran negative (Fig. 3-3C), which implied that the particles were taken up predominantly through phagocytosis. Membrane staining performed with Alexa Fluor® 488 gave better visualization of PLGA nanoparticle uptake with respect to DC surface and confirmed the internalization and intracellular localization of the nanoparticles through visualization of DCs at different planar sections (Fig. 3-4).

3.3.5 Uptake of PLGA nanoparticles by DCs is not mediated through mannose receptors (MRs)

DCs express MRs, which play an important role in receptor mediated endocytosis of molecules containing mannose or mannose like moieties such as dextran (6, 7). During the preparation of TMR-dextran containing PLGA nanoparticles, some of the fluorescent probe may get localized on the surface of the formulation allowing MRs on DCs to engage the nanoparticles and mediate the uptake process. This possibility was

investigated by performing uptake studies in the presence of soluble dextran and mannose that would compete for MRs. Pre-incubation of DC cultures with either mannose or dextran prior to the addition of PLGA nanoparticles showed only a minor reduction in the extent of nanoparticle uptake (Fig. 3-5). The uptake of soluble TMR-dextran was clearly inhibited in presence of these competitive inhibitors. For the sake of clarity, the inhibitory effects on a five fold higher concentration of soluble TMR-dextran are depicted in Figure 3-5. These observations indicated that TMR-dextran when in solution form was internalized through MR mediated endocytosis whereas the uptake of PLGA nanoparticles containing TMR-dextran was MR independent. The almost complete arrest of PLGA nanoparticle uptake by Cytochalasin B pre-treatment (Fig. 3-5) further confirmed that phagocytosis is the primary mechanism of nanoparticle uptake by DCs.

3.3.6 Encapsulation of antigens in PLGA nanoparticles is superior to solution form for delivering the dose of the antigen to DCs

The amount of administered dose of the antigen that is taken up by DCs when encapsulated in PLGA nanoparticles or in solution form was assessed by incubating immature DC primary cultures with ^{125}I-BSA either in free form or encapsulated in PLGA nanoparticles. Twenty fours hours later, pure DC populations from the cultures were isolated by positive selection using anti CD11c magnetic beads and the radioactivity incorporated inside the DCs counted. As depicted in Fig. 3-6, DCs had taken up 2.5% of the total administered antigen dose when ^{125}I-BSA was encapsulated in PLGA nanoparticles. Less than 0.1% of the total delivered dose was taken up when the antigen was administered in solution form. When the dose of ^{125}I-BSA in solution form was

increased by 100 fold, 0.6% of the total administered dose of the antigen was taken by DCs. These results confirmed that delivery of antigen in PLGA nanoparticles was superior in delivering the load of the encapsulated antigen into DCs and this mode of antigen delivery is superior to the soluble form for uptake by DCs.

3.4 Discussion

The current set of experiments were performed to characterize a biodegradable particulate antigen delivery system fabricated using the PLGA co-polymer for delivery of vaccine antigens to DCs for applications in DC immunotherapy. A MUC1 lipopeptide antigen was formulated in PLGA nanoparticles and this formulation was proposed to be a promising cancer vaccine formulation for cancer immunotherapy (Chapter 2). The potential of this nanoparticulate delivery system for delivering other MUC1-based cancer vaccines, hepatitis B antigens, and other antigens using both *in vitro* and *in vivo* models has been described (8-10).

Flow cytometry was employed in the initial experiments as this technique separates free nanoparticles from DCs during acquisition based on the size and granularity of the cells. It also offers the feasibility to generate quantitative information like determination the number of cells taking up the nanoparticles and the extent of particle uptake per cell. Fluorescent PLGA nanoparticles (TMR-dextran nanoparticles) allowed the employment of dual color flow cytometry to positively identify the phagocytic population in the culture on the basis of cell surface marker expression.

The current studies demonstrated that an appreciable number of *in vitro* generated DCs internalized PLGA nanoparticles and antigens encapsulated in PLGA nanoparticles

are efficiently delivered to DCs. As macrophages are a minor contaminant population in the generated DC primary cultures and have the ability to take up micro and nanoparticles (11, 12), an anti CD11c mAb which is a DC specific marker was employed to identify the DC population. The results indicated that the cells that had internalized the nanoparticles were primarily DCs and these cells were also MHC class II and CD86 positive.

The maturation stage plays a critical role in the ability of DCs to take up PLGA nanoparticles. In order to determine the ideal timing for delivering PLGA nanoparticles to DCs to support future DC experiments, the optimal culture time of the propagated DC primary cultures to internalize PLGA nanoparticles was determined. The difference in uptake of PLGA nanoparticles between 6 to 8 day old DC cultures was fairly low and not statistically significant ($P > 0.05$). However, day 9 DC cultures had a slightly lower nanoparticle uptake ability than the earlier cultures and this phenomenon may be attributed to maturation induced over prolonged time in culture (described in Chapter 4). The dose of the nanoparticles added to the culture media also influenced the extent of nanoparticle uptake by DCs as doses up to 25-50 μg/mL (0.5 to 1 mg/20 mL) were moderately taken up over a 24 h period whereas the threshold for the number of DCs that internalized the nanoparticles was reached at 100 μg/mL. However, increasing the treatment dose beyond 100 μg/mL continued to enhance the MFI of the fluorescent nanoparticles within the DCs. This data indicated that most of the generated DCs in the culture internalized the nanoparticles and participated in the uptake process while increasing the nanoparticle treatment dose beyond 100 μg/mL allowed DCs to

phagocytose additional particles. Similarly, the uptake of nanoparticles was also influenced by the incubation time with maximal levels attained by 24 h.

Since dextran is similar to mannose in structure, there may be possibility for the observed uptake of TMR-dextran containing PLGA nanoparticles to be mediated through MRs. However, the failure of free mannose or dextran to inhibit PLGA nanoparticle uptake suggested that MR mediated endocytosis was not involved in the uptake process whereas phagocytosis was the key mechanism due to inhibition of uptake in presence of Cytochalasin B (13).

As the goal of these studies was to assess the efficiency of targeting antigens encapsulated in PLGA nanoparticles to DCs for generation of cellular immune responses, it was pivotal that the nanoparticles be localized in the intracellular compartments after uptake. As flow cytometry cannot distinguish between extracellular (surface adsorbed) and intracellular fluorescence, CLSM studies were performed to pin-point the location of the phagocytosed fluorescent nanoparticles. The data from the CLSM studies indicated that the PLGA nanoparticles are indeed taken up by DCs and localized in their intracellular compartments.

In vitro generated DCs took up approximately 2.5% of the total antigen dose administered when the antigen was encapsulated in PLGA nanoparticles whereas less than 0.05% of the treated dose was taken up when the antigen was presented in the solution form. It can be hypothesized that soluble molecules are indeed taken up by DCs though at lower amounts and due to their physical nature probably get chewed up and removed from the cell limiting their intracellular concentrations. However, antigens encapsulated in PLGA nanoparticles are efficiently taken up due to their particulate

nature and are slowly released into the phagosome or endosome through degradation of the polymeric matrix. This controlled and sustained pattern of antigen release within the DCs will maintain adequate physiological concentrations of the antigen for prolonged loading onto MHC molecules leading to enhanced presentation of the processed antigen. Audran et. al. have demonstrated that microparticle delivery prolonged the antigen presentation of DCs for up to seven days(14).

Efficient delivery of antigens to *ex vivo* generated DCs is an important aspect for exploiting the potential of DCs as cellular vaccines for application in immunotherapy of cancer. The current data strongly indicated that PLGA nanoparticles serve as efficient antigen delivery vehicles for delivering the load of the antigen into DCs. These observations thus lay the platform for future studies directed at assessing the various aspects of antigen and adjuvant delivery to DCs employing PLGA nanoparticles.

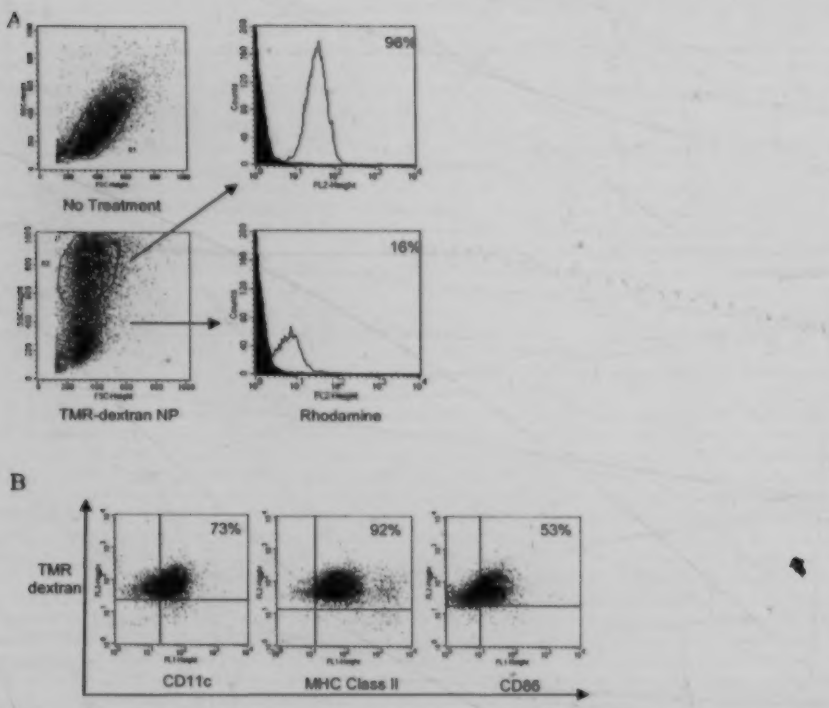

Figure 3-1. Flow cytometric analysis of DCs after treatment with fluorescent PLGA nanoparticles. Day 7 DC primary cultures were incubated with TMR-dextran containing PLGA nanoparticles for 24 h and analyzed by FACS. (A) FSC and SSC profiles of unpulsed and nanoparticle pulsed DCs. Black histogram represents background fluorescence obtained following empty PLGA nanoparticle treatment. Two colour FACS dot plots gated on the live high granularity cell populations indicating the phenotype of cells that had taken up the nanoparticles (B). A representative of four independent experiments is shown.

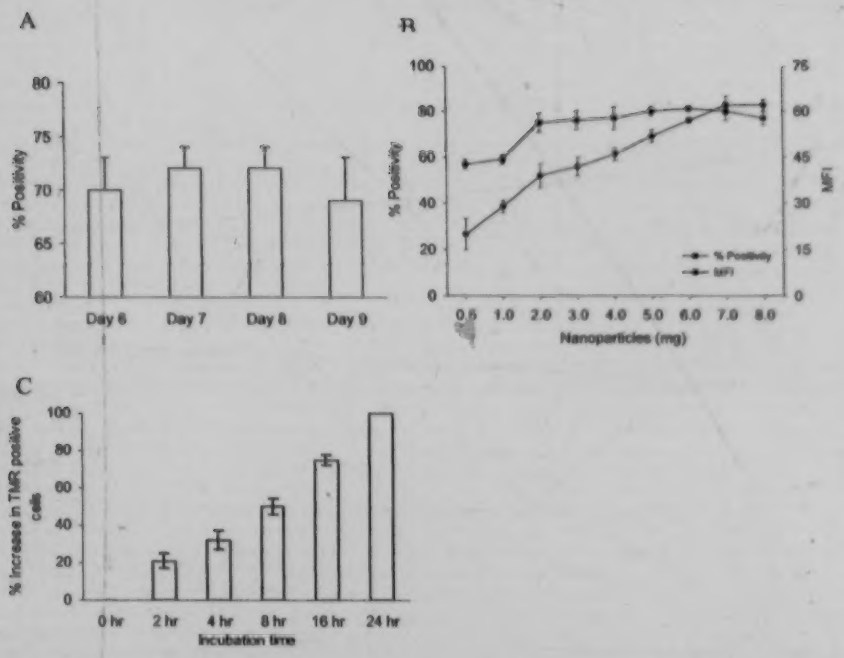

Figure 3-2. Experimental conditions influencing the uptake of TMR-dextran containing PLGA nanoparticles by DCs. DC primary cultures on various days of propagation were incubated with various concentrations of TMR dextran containing PLGA nanoparticles and incubated for different times. The influence of experimental conditions, namely- the age of the DC primary culture for uptake of 100 μg/mL of nanoparticles (A), nanoparticle concentration- uptake of PLGA nanoparticles by day 7 DCs (B), and incubation time- day 7 DCs incubated with 100 μg/mL of PLGA nanoparticles (C) is shown. Error bars represent standard deviation between samples in triplicates. One representative experiment out of two is shown.

Figure 3-3. CLSM images of uptake of PLGA nanoparticles by DCs. Day 7 DC primary cultures were incubated on chamber slides with (B) or without (A) TMR-dextran containing PLGA nanoparticles (50 μg/mL) for 24 h and stained using CD11c mAb and FITC conjugated second mAb. Inhibition of uptake was demonstrated by pretreatment of DC cultures with Cytochalasin B for 30 min prior to addition of PLGA nanoparticles (C). White bar represents 15 μm. One representative experiment out of two is shown.

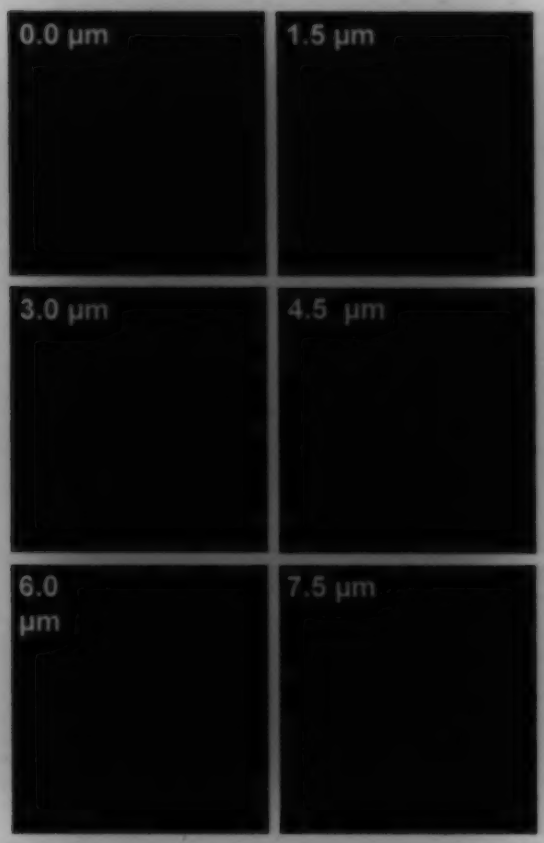

Figure 3-4. Intracellular localization of fluorescent PLGA nanoparticles after uptake by DCs. Day 7 DC primary cultures incubated with TMR-dextran containing PLGA nanoparticles were stained with Alexa Fluor® 488 and analyzed by CLSM at different planar sections. One representative experiment out of two is shown.

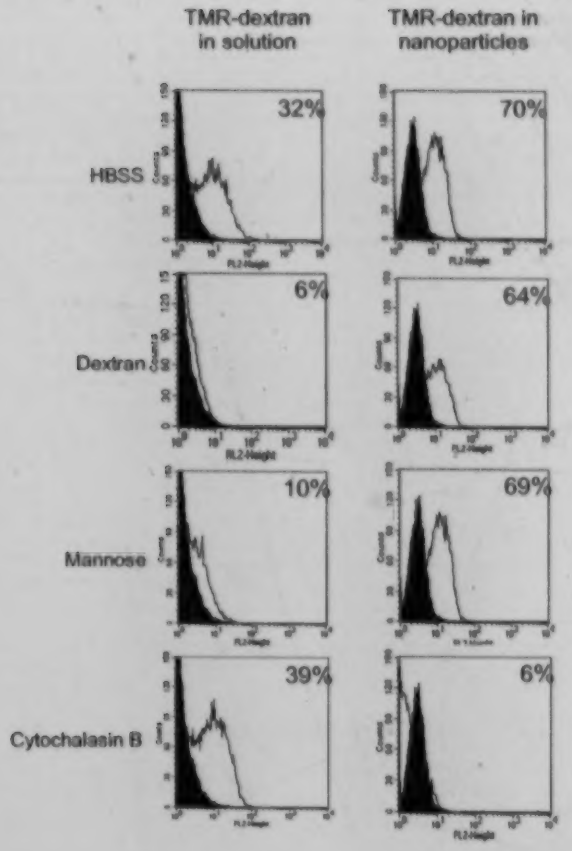

Figure 3-5. Analysis of mechanism of uptake of PLGA nanoparticles by DCs. DC primary cultures were pre-treated with either HBSS or mannose or dextran or Cytochalasin B for 30 min prior to the addition of TMR-dextran containing formulations. The extent of uptake was analyzed by FACS. Solid black areas in the histogram plot indicate the background fluorescence after pulsing the cells either with empty nanoparticles (for nanoparticle uptake studies) or HBSS (for soluble formulation uptake studies). One representative experiment out of two is shown.

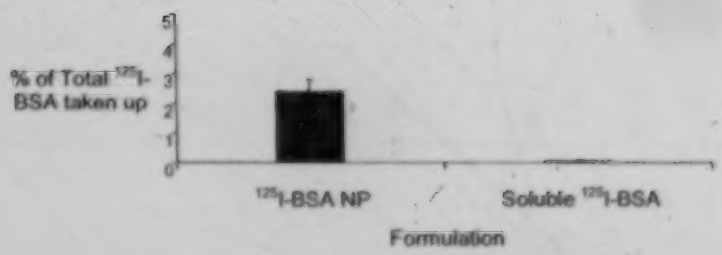

Figure 3-6. Analysis of the dose of antigen taken up by DCs when delivered in free form or in PLGA nanoparticles. ^{125}I-BSA either in PLGA nanoparticles or in solution was incubated with DC primary cultures for 24 h. After co-culture, pure DC populations were harvested by positive selection and the radioactivity incorporated counted and compared against the total radioactivity added. Error bars indicate S.D. between samples analyzed in triplicates. One representative experiment out of two is shown.

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Chapter Four

Poly(D,L-lactic-co-glycolic acid) nanoparticle mediated delivery of Monophosphoryl lipid A and antigen to DCs for inducing enhanced maturation and generating potent antigen specific primary T cell responses *in vitro* and *in vivo*.

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4.1 Introduction

The maturation status of dendritic cells (DCs) plays a key role in the outcome of the immune responses where immature DCs cause T cell anergy, deletion of autoreactive T cells, and regulatory T cell induction whereas as fully mature DCs are involved in immune cell activation (1, 2). A number of external stimuli induce DC maturation (discussed in chapter 1). Monophosphoryl lipid A (MPLA) is a detoxified product of lipopolysaccharide (LPS) that is found in the outer membranes of gram negative bacteria. The removal of the phosphate and fatty acid groups from lipid A of LPS generates MPLA that retains the adjuvant property but not the associated toxicity (3). This detoxified form of LPS i.e. MPLA activates DCs through Toll like receptor 4 (TLR4) and has been studied for its potential in modulating immune responses (4, 5). It has proven potential as an effective vaccine adjuvant in human clinical trials for cancer, infectious diseases, and allergy and is well tolerated with a safety profile similar to Alum making it a clinically promising adjuvant (6).

The purpose of these series of studies was to systematically characterize the maturation following delivery of MPLA encapsulated in Poly (D,L lactic-co-glycolic acid) (PLGA) nanoparticles to DCs. Furthermore, the induction of primary T cell immune responses by DCs following nanoparticle mediated delivery of a model antigen-OVA (ovalbumin) was investigated. As cancer antigens are self antigens and immune recognition and overcoming self tolerance is a major limitation in cancer immunotherapy, the ability of DCs to overcome self tolerance mechanisms against MUC1 following the particle mode of antigen (MUC1) delivery was assessed using T cells derived from

human MUC1 transgenic mice (MUC1. Tg mce) where MUC1 is expressed as a self antigen.

4.2 Materials and Methods

4.2.1 Mice

Balb/c mice and C57BL/6 mice were bred and maintained at the University of Alberta's Health Science's Laboratory Animal services (HSLAS). Male C57BL/6 mouse strain transgenic for human MUC1 (MUC1.Tg) was kindly provided by Dr. Sandra Gendler (Mayo Clinic, Scottsdale, AZ) and bred at the University of Alberta's transgenic animal breeding facility at the HSLAS. Polymerase chain reaction (PCR) sample amplification from DNA extracted from the ear biopsies using MUC1 primers (bp745-765 and bp1086-1065) (Biomira Inc., Edmonton, AB, Canada), was used to routinely identify MUC1 positive mice in the colony. MUC1 expression in various organs and tissues from selected mice was analyzed by immunohistochemical staining with mAb against MUC1 antigen (7). DO11.10 TCR transgenic mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All experiments were performed in accordance to the University of Alberta's guidelines for the care and use of laboratory animals. All experiments were performed using 8 to 10 week old male mice.

4.2.2 Reagents and antibodies

Human MUC1 lipopeptide- (STAPPAHGVTSAPDTRPAPGSTAPP-Lysine(palmitoyl)G) (BLP25) and MPLA, m.w., 1955.5 Da were kindly provided by Biomira Inc., (Edmonton, AB, Canada). Chicken ovalbumin (OVA) protein (Grade-V),

LPS, and polyvinyl alcohol (PVA, m.w 21-50,000 Da) were obtained from Aldrich Chem (Milwaukee, WI). Tetramethylrhodamine-dextran (TMR-dextran, m.w., 40,000 Da) was purchased from Molecular probes (Eugene, OR). PLGA co-polymer, monomer ratio 50:50, m.w 7000 Da, was purchased from Birmingham polymers (Birmingham, AL). Recombinant murine granulocyte macrophage colony stimulating factor (rmGM-CSF) was purchased from Peprotech (Rockville, IL). Anti mouse CD16/32, CD11c, MHC class II, CD86, and CD40 mAbs, apoptosis detection kit (Annexin V-FITC and Propidium Iodide), Cytofix/Cytoperm kit with Golgi-stop, and the murine IL-12p70 ELISA kit were purchased from BD Biosciences (Mississauga, ON, Canada). TranSignal™ mouse cytokine antibody array membranes were purchased from Panomics Inc (Redwood City, CA). Murine CD11c and CD4 isolation kits and the CD90.2 mAb were purchased from Stem Cell Technologies (Toronto, ON, Canada). Phycoerythrin conjugated anti mouse CD4, IL-2, IFN-γ, CD11a, CD44, CD62L, and CD69 mAbs and their respective isotype controls and murine IL-6 and TNF-α ELISA kits were purchased from E-Biosciences (San Diego, CA). Clonotype specific DO11.10 TCR transgenic mAb, KJ1-26 and its isotype control were purchased from Caltag Laboratories (Burlingame, CA). QCL-1000 Limulus Amebocyte Lysate (LAL) endotoxin detection kit was purchased from BioWhittaker (Walkersville, MD). RPMI-1640, L-glutamine, and gentamycin were purchased from Gibco-BRL (Burlington, ON, Canada). Fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT).

4.2.3 Preparation of PLGA nanoparticles

Single emulsion PLGA nanoparticles containing BLP25 with or without MPLA were prepared as described earlier in chapter 2. PLGA nanoparticles containing OVA protein with or without MPLA were prepared as water/oil/water double emulsion formulation by the solvent evaporation method. Briefly, OVA protein [0.05-50 µL, 100 mg/mL solution in PBS (pH-7.4)] was emulsified with PLGA solution in chloroform (200 µL, 50% w/v) using a microtip sonicator (Heat systems Inc., Farmingdale, NY). For preparation of nanoparticle containing MPLA, 25-200 µg of MPLA in 100 µl of 1:4 methanol-chloroform mixture was added to the polymer-chloroform solution. The resulting primary emulsion (water/oil) was further emulsified in 2 mL of PVA solution [9% w/v PVA in PBS (pH-7.4)] by sonication for 45 sec at level 4. The secondary emulsion was transferred drop-wise into a beaker containing stirring 8 mL of PVA solution. Nanoparticles were collected after 2.5 h of stirring by centrifugation of the emulsion at 17,000 rpm for 10 min at 4°C and washed twice with cold deionized water and freeze-dried. OVA loading in PLGA nanoparticles was determined by Bicinchoninic acid (BCA) protein assay and by reverse phase HPLC for BLP25. The mean hydrodynamic diameter of the nanoparticles ranged between 350-450 nm with a polydispersity below 0.1 as determined by dynamic light scattering methodology. MPLA loading in PLGA nanoparticles was determined as described in Chapter 2.

4.2.4 Endotoxin Testing

One milligram of empty PLGA nanoparticles was suspended in endotoxin free water and shaken for 6 h at 37°C. The nanoparticle suspension was centrifuged at 14000 rpm

for 10 min and the supernatant tested for presence of endotoxin using the QCL-1000 LAL endotoxin testing kit according to the manufacturer's directions. An extremely low amount of endotoxin (below 0.1 EU/mL) was present in the supernatant of the nanoparticle formulation which was well below the acceptable United Sates Pharmacopeia (USP) standard for water for injection (less than 0.25 EU/mL).

4.2.5 Cell culture

Murine DC primary cultures were generated from bone marrow precursors obtained from femurs of C57BL/6 or Balb/c mice in complete media (RPMI-1640 medium, L-glutamine, and gentamycin) supplemented with 10% FCS and 20 ng/mL of rmGM-CSF as described in chapter 3.

4.2.6 Treatment of DC cultures with antigen and adjuvant formulations

On day 7 of the murine DC primary culture, the semi-adherent and non-adherent cell populations were harvested and replated at 2 or 5 × 10^5 cells/mL in 50 mm bacteriological grade petri dishes in complete media with 20 ng/mL of rmGM-CSF. Soluble or nanoparticle antigen-adjuvant formulations were incubated for up to 24 h with the DC cultures. For some maturation studies, after 24 h of formulation treatment, DCs were harvested and washed thoroughly and further propagated for up to 7 days. For cytokine and chemokine secretion studies, pure DC populations from non-adherent and semi-adherent cells of day 7 DC primary cultures were isolated by positive selection using the murine CD11c isolation kit according to the manufacturer's protocol. Purity of the positively isolated DCs was > 95%. The positively selected DCs were replated at 2 ×

10^5 cells/mL in complete media with or without supplemented rmGM-CSF and treated with various formulations for up to 48 h.

4.2.7 Flow cytometric analysis

For phenotypic maturation studies, 2.5×10^5 harvested DC populations were suspended in FACS buffer (PBS with 5% FCS, and 0.09% sodium azide) and incubated with anti-CD16/CD32 mAb to block Fc receptors and then stained with MHC class II, CD86, and CD40 mAbs followed by incubation with appropriate fluorescence conjugated second step antibodies or reagents. For intracellular cytokine analysis, DC and T cell co-cultures were treated with Golgi-Stop for 10 h and stained with anti CD90.2 and KJ1-26 mAbs. The cells were then permeabilised with Cytofix/Cytoperm buffer and stained with anti-cytokine mAbs. Samples were acquired on a Becton-Dickinson FACSort (BD Biosciences, Franklin Lakes, NJ) by gating on the live cell populations and analyzed by Cell-Quest software.

4.2.8 Allogeneic mixed lymphocyte reaction (MLR)

Two hundred and fifty thousand nylon wool purified T cells obtained from spleens of Balb/c mice were co-cultured with irradiated DCs (generated from C57BL/6 mice) that were either untreated or treated with soluble or particulate formulations at different DC and T cell ratios. The co-cultures were conducted in complete media in presence of 5% mouse serum in 96 well flat bottom plates at 37°C and 5% CO_2. [^{3}H]-thymidine was added during the last 18 h of a four day co-culture and the T cell proliferation measured by [^{3}H]-thymidine incorporation.

4.2.9 Cytokine and chemokine assays

Supernatants of positively isolated murine DC cultures ($CD11c^+$) that were treated with PBS or soluble or nanoparticle formulations containing MPLA for 6 to 24 h (dose of MPLA- 100 ng/mL) were screened for presence of cytokines and chemokines by the TranSignal™ mouse cytokine antibody array membranes following manufacturer's directions. The sensitivity of the assay ranged between 800-1000 pg/mL as indicated by the manufacturer. Quantitative cytokine analysis for IL-6, IL-12p70, and TNF-α in the culture supernatant was performed by ELISA kits in a 96 well microplate using a microplate reader (Powerwave with KC Junior software; Bio-Tek, Winooski, VT) at OD of 450 nm with reference set at 570 nm according to the manufacturer's directions. All samples were analyzed in triplicates. The minimum detection levels for the cytokines were: IL-12p70, 5 pg/mL; IL-6, 10 pg/mL; TNF-α, 15 pg/mL).

4.2.10 Cellular viability studies

The influence of soluble or nanoparticulate MPLA formulations on DC viability and toxicity was studied by the AnnexinV-FITC/Propidium iodide staining according to the manufacturer's directions. Briefly, 2×10^5 DCs treated with PBS or MPLA formulations for 24 h were incubated with AnnexinV-FITC and Propidium iodide for 30 min and analyzed by two colour flow cytometry.

4.2.11 DO11.10 CD4 T cell isolation, *in vitro* T cell activation/proliferation, and intracellular cytokine analysis

$CD4^+$ T cells from the spleens of DO11.10 mice were isolated by negative selection using the Easy Sep mouse CD4 T cell isolation kit according to the manufacturer's instructions. The purity of the isolated $CD4^+$ T cells was > 95% and greater than 85% of these $CD4^+$ T cells stained positive for the clonotype specific KJ1-26 mAb. Antigen or PBS treated DCs were harvested, irradiated (3000 rads), washed, and co-cultured (at 5×10^4 DCs) with 2.5×10^5 $CD4^+$ DO11.10 T cells per well in a 96 well flat bottom tissue culture plate. Intracellular cytokine analysis for IL-2 and IFN-γ secretion by $CD4^+$ T cells was performed after 36 h of co-culture. [^{3}H]-thymidine was added during the last 18 h of a 60 h co-culture for measurement of T cell proliferation. In some experiments, DC-T cell co-cultures were harvested from the culture plate after 72 h and the expression of CD44, CD62L, and CD69 on the live $CD4^+$/KJ-126^+ T cells determined by flow cytometry.

4.2.12 *In vivo* adoptive transfer and DC immunization

Five million $CD4^+$ T cells isolated from spleens of DO11.10 mice were adoptively transferred into Balb/c mice by i.v. injections in the tail vein. After resting for 24 h, the recipient mice were vaccinated i.p. with 1×10^6 DCs that were pre-treated with various OVA and MPLA formulations for 12 h. DCs were pulsed *in vitro* with OVA either in solution form or in PLGA nanoparticles at 5 μg/ml for all *in vivo* experiments. Control mice received DCs incubated with PBS. Seventy two hours following vaccination, spleens of the vaccinated mice were harvested and single cell suspensions prepared and blocked with 0.1 μg of CD16/32 mAb. For antigen specific T cell clonal expansion

studies, the spleenocytes were incubated with 0.125 µg of CD4 and KJ1-26 mAbs and the percent of live $CD4^+/KJ1\text{-}26^+$ cells determined by flow cytometry. For T cell activation/memory studies, spleenocytes were stained with 0.125 µg of CD90.2 and KJ1-26 mAbs followed by staining with one of the fluorescence conjugated CD11a, CD44, and CD62L mAbs.

4.2.13 *In vitro* T cell proliferation studies using a therapeutically relevant cancer antigen

DCs generated from wild-type or MUC1.Tg mice were incubated with either of the following antigen formulations for 24 h: (a) nanoparticles containing BLP25 and MPLA: [BLP25+MPLA] NP (b) nanoparticles containing BLP25: [BLP25] NP (c) nanoparticles containing MPLA: [MPLA] NP (d) empty nanoparticles [Empty] NP and (e) soluble BLP25 and MPLA (Sol.BLP25/MPLA). After incubation, DC cultures were harvested, irradiated and washed thoroughly and seeded in triplicates in 96 well flat bottomed microtiter plates (Costar, Cambridge, MA) in complete RPMI. Nylon wool purified T cells from either wild-type or MUC1.Tg mice were co-incubated at 2×10^5 cells per well with DC at ratios of 1:5 and 1:10. After 72-96 h of incubation, the cell proliferation was assessed by [^{3}H]-thymidine incorporation following a final 24 h pulse.

4.2.14 Statistical analysis

The results are expressed as the mean ± standard deviation (S.D.) for each treatment group. An unpaired student's 't-test' was used to compare sample means and assess statistical significance. Results were considered significant if $P < 0.05$.

4.3 Results

4.3.1 Phenotypic maturation of DCs following MPLA stimulation

The capability of PLGA nanoparticles to serve as antigen delivery vehicles was demonstrated in Chapter 3. In the current study, the PLGA nanoparticle delivery system was evaluated for targeting a promising adjuvant- MPLA to DCs to induce DC maturation. MPLA in free form (in solution) has been previously reported to induce a moderate maturation in human DCs, although this required doses as high as 50-100 μg/mL (8). In the current study, the extent of phenotypic maturation of murine DCs following delivery of MPLA either in PLGA nanoparticles or in free form at a lower dose (100 ng/mL) was systematically evaluated based on the surface expression of MHC class II, CD86, and CD40 molecules. DCs that were treated with PBS alone were MHC Class II^{high}, $CD40^{low}$, and $CD86^{low}$ (Fig. 4-1). Both the modes of MPLA treatment at 100 ng/mL upregulated the expression of MHC class II to a similar extent (Fig. 4-1). A 2.5 fold increase in the number of DCs expressing CD86 was observed following delivery of MPLA in PLGA nanoparticles as compared to a 2 fold increase observed with MPLA in solution (free MPLA). A two fold increase in the percent of DCs expressing CD40 was observed following MPLA delivery either in solution form or in PLGA nanoparticles However, a 14 fold increase in the mean fluorescent intensity (MFI) of CD40 expression following treatment with soluble MPLA was observed (Fig. 4-1). Furthermore, treatment with MPLA nanoparticles resulted in greater upregulation of CD86 and CD40 as compared to LPS stimulation. Empty nanoparticle treatment induced an intermediate increase in expression of maturation molecules probably due to an activation stimulus triggered by phagocytosis and not due to endotoxin contamination, since nanoparticles

were tested for endotoxin and shown to be free from contamination. The mature phenotype of DCs was also retained for up to seven days following treatment with MPLA containing nanoparticles (Fig. 4-2). The viability of these propagated DCs was greater than 80% as observed by trypan blue exclusion before staining with mAbs for flow cytometry. Except for MHC class II whose MFI continued to rise over time in culture, the MFI of CD86 and CD40 gradually increased for up to 48 h following MPLA nanoparticle treatment and then slightly declined (Fig. 4-2).

4.3.2 DCs demonstrate an enhanced allostimulatory capacity following treatment with MPLA containing nanoparticles

Having observed that phenotypic maturation following particulate delivery of MPLA to DCs was efficiently achieved, the functionality of these activated DCs was assessed in a primary MLR. Following treatment with MPLA nanoparticles (at 100 ng/mL of MPLA) for 12 h, $CD11c^+$ DCs were positively isolated, irradiated, and co-cultured at different ratios with nylon-wool purified T cells obtained from the spleens of Balb/c mice. As depicted in Fig. 4-3, DCs stimulated with MPLA in either soluble or particulate form or with LPS generated high allogeneic T cell proliferative responses (at 1:10 ratio) than control DCs. However, DCs matured with MPLA nanoparticles were more functional as the extent of T cell stimulation was significantly greater when the number of available stimulator cells was low i.e. at 1:50 and 1:100 ratios (statistical significance between samples of MPLA nanoparticle treatment and soluble MPLA, $P < 0.05$). Empty PLGA nanoparticle treatment moderately enhanced the ability of DCs to stimulate allogeneic T

cells. These results indicated that delivery of MPLA in PLGA nanoparticles improved the functionality of DCs in a comparable level to that observed following LPS treatment.

4.3.3 DCs secrete high levels of Th1 promoting cytokines and chemokines following treatment with PLGA nanoparticles containing MPLA

Fully mature and functional DCs secrete pro-inflammatory cytokines and chemokines in response to a danger signal (9). In this context, the secretion of cytokines and chemokines by DCs following delivery of MPLA in PLGA nanoparticles was assessed using a membrane bound antibody array system that screened the DC culture supernatant for 18 different proteins which included various Type I and II immune response polarizing cytokines and chemokines. As shown in Fig. 4-4, none of the proteins being screened were detected in the supernatant of PBS treated DC cultures except for low levels of macrophage inflammatory protein-1 alpha (MIP-1α), regulated on activation normal T cell expressed and secreted (RANTES), and GM-CSF. The cytokine/chemokine secretion profile of DC cultures treated with soluble MPLA was similar to that of PBS treated DC cultures. Treatment of DC cultures with MPLA encapsulated in PLGA nanoparticles induced high levels of secretion of pro-inflammatory cytokines and chemokines namely IL-6, IL-12p40/70, tumour necrosis factor-alpha (TNF-α), MIP-1α, and RANTES and intermediate levels of G-CSF and GM-CSF and low levels of IFN-γ, IL-1α, IL-4, and IL-13. One pilot study further indicated that empty PLGA nanoparticles treatment generated the same kind of cytokine and chemokine secretion profile as observed following plain media treatment. Therefore these results indicated that delivery of MPLA in PLGA nanoparticles to DCs induced the secretion of Th1 polarizing

cytokines and chemokines and also implied that MPLA was physically required to be present in the nanoparticle formulation for efficient DC stimulation. The kinetics of IL-6, IL-12p70, and TNF-α secretion by DCs following treatment with MPLA containing formulations was next assessed. As show in Fig. 4-5, IL-12p70 and IL-6 were detected within 6 h of MPLA nanoparticle treatment and continued to rise though at a low rate over 24 h. On the other hand, secretion of TNF-α peaked within 6 h of treatment and remained sustained for up to 24 h. Cytokine release was also found to be dependent on the dose of MPLA in the nanoparticles as a very low amount of cytokine was detected following treatment of DC cultures with either 12.5 or 25 ng/mL of MPLA in PLGA nanoparticles whereas 50 and 100 ng/mL MPLA doses secreted similar levels of the cytokines (Fig. 4-6). A two fold decrease in cytokine secretion upon treatment of DC cultures with MPLA nanoparticles in the absence of GM-CSF was also observed (Fig. 4-7). Furthermore, treatment of DC cultures with empty nanoparticles or OVA containing nanoparticles did not induce secretion of any detectable amount of IL-12p70. These observations that suggested that empty nanoparticle treatment does not cause any cytokine secretion is consistent with the fact that the prepared PLGA nanoparticles are free of any endotoxin contamination.

4.3.4 Treatment of DCs with MPLA nanoparticles does not induce cellular apoptosis

Although PLGA is a biodegradable and non-toxic polymer, its presence at high concentrations inside the cell may lead to cellular toxicity. Also, the degradation products of PLGA i.e. lactic and glycolic acids, which normally are metabolized through the citric acid cycle, may be toxic to the cell if present at high concentrations due to the highly

acidic pH environment that is generated. Therefore, to assess for induction of cell death via apoptosis to DCs following nanoparticle mode of MPLA delivery, Annexin V-FITC/PI staining was performed on PBS or soluble or nanoparticle MPLA formulation treated DC cultures. As shown in Fig. 4-8, only a minor difference in cell death/apoptosis above the normal basal level observed following treatment with PBS was detected in DC cultures treated with either MPLA nanoparticles or soluble MPLA (statistical significance between MPLA nanoparticle and soluble MPLA treatment when compared to PBS treatment, $P > 0.05$). Likewise, empty nanoparticle treatment too was not associated with any undue toxicity to DCs (statistical significance between empty nanoparticle and PBS treatment, $P > 0.05$). Increasing the treatment dose of MPLA containing nanoparticles from 50 to 200 μg/mL (concentration of nanoparticles in the culture) augmented the number DCs undergoing apoptosis whereas a similar pattern of decreased viability was not observed with higher doses of soluble MPLA. These observations indicated that higher doses of polymeric nanoparticles and not the MPLA inside the cell most likely induced toxicity. Thus, though the delivery of MPLA in PLGA nanoparticles to DCs induced a powerful maturation process, this mode of stimulation is relatively safe and has a very minimal impact on DC viability at the concentration of the polymer being employed in the current studies.

4.3.5 Co-delivery of antigen and MPLA in PLGA nanoparticles to DCs is more effective than their delivery in soluble form for primary activation of $CD4^+$ T cells *in vitro*

After having observed that the delivery of MPLA in PLGA nanoparticles superiorly induced phenotypic and functional maturation of DCs, the applicability of the PLGA nanoparticulate delivery system for targeting antigens along with MPLA into DCs for the subsequent generation of antigen specific primary T cell immune responses was assessed. Chicken OVA protein was used as the model antigen for these studies. T cells obtained from DO11.10 mice which express T cell receptor (TCR) that recognize $OVA_{(323-339)}$ in context of $I\text{-}A^d$ were employed for assessing the T cell responses following *in vitro* DC vaccination. As illustrated in Fig. 4-9, DCs incubated with soluble OVA protein at doses of up to 100 ng/mL did not prime antigen specific T cells. Similarly, co-incubation of MPLA along with OVA in solution form with DCs did not result in any detectable proliferation of OVA specific T cells. However, delivery of OVA protein encapsulated in PLGA nanoparticles to DCs significantly primed naïve T cells in an antigen specific and dose dependent manner. Furthermore, incorporation of MPLA along with OVA protein in the PLGA nanoparticle formulation significantly increased the extent of T cell stimulation when compared to OVA nanoparticle treatment alone at antigen doses of 10 and 100 ng/mL. These results demonstrated that the PLGA nanoparticle mode of antigen delivery augments the ability of DCs to enhance the immune responses against a particular antigen. Similarly, the nanoparticle mode of antigen delivery decreases the effective dose of the soluble antigen needed to induce a strong primary T cell immune response. Furthermore, incorporation of TLR ligands like MPLA in the nanoparticle

formulation can increase the T cell activation potential of DCs and at the same time decrease the antigen dose necessary for superior T cell priming.

4.3.6 T cells primed by DCs treated with antigen-MPLA nanoparticles secrete Th1 promoting cytokines and exhibit an activated and memory phenotype

Secretion of IL-2 and IFN-γ by T cells is indicative of the development of Th1 type immune responses. The secretion of these two key cytokines by antigen specific T cells in the *in vitro* T cell proliferation culture was studied by intracellular cytokine staining. To identify the DO11.10 $CD4^+$ T cell population in the co-culture, the KJ1-26 antibody was employed that binds to the OVA-specific TCR (10). As shown in Fig. 4-10, DCs pulsed with OVA protein containing nanoparticles efficiently stimulated a large number of $CD90.2^+/KJ1\text{-}26^+$ T cells to secrete IL-2 and IFN-γ. These cytokines were being secreted by the proliferating and not unstimulated or resting T cells as the cytokine secreting T cells had an increased cell size which indicated the blast formation stage of cell division. The background secretion of both of these cytokines by T cells following incubation with unpulsed DCs was below 10%. Consistent with the *in vitro* T cell proliferation data, co-delivery of MPLA in PLGA nanoparticles along with OVA increased the number of T cells secreting IL-2 and IFN-γ ($P<0.05$, between PBS treatment and OVA NP or OVA/MPLA NP treatment). This increase in the number of T cells secreting these two cytokines was probably being mediated through the strong adjuvant effect of MPLA via secretion of IL-12 by DCs. Furthermore, when the treatment dose of the OVA (encapsulated in PLGA nanoparticles) was decreased by ten fold (from 100 to 10 ng/mL) the number of T cells secreting the cytokines still remained higher

when MPLA was present in the nanoparticle formulation containing OVA. In the next step, the expression of activation and memory markers on the primed T cells was analyzed. OVA specific T cells primed by DCs targeted with OVA protein in PLGA nanoparticles were $CD44^{hi}CD62L^{lo}CD69^{hi}$ (Fig. 4-11). The presence of MPLA in the OVA containing nanoparticle formulation did not have a profound effect on the intensity of expression of these surface activation and memory markers. These results demonstrated that DCs pulsed with antigens encapsulated in PLGA nanoparticles primed T cells in a manner where naive T cells were transformed into activated Th1 cytokine secreting effector T cells and memory T cells.

4.3.7 DCs pulsed *in vitro* with antigen and MPLA encapsulated in PLGA nanoparticles activate and clonally expand antigen specific T cells *in vivo*

The generation of potent antigen specific T cell immune responses *in vitro* following nanoparticle mode of antigen delivery to DCs (4.3.5 and 4.3.6) prompted for the validation of this mode of cellular vaccine delivery for *in vivo* applications. The induction of primary T cell immune responses *in vivo* was assessed using an adoptive transfer model. In these experiments, $CD4^+$ T cells from DO11.10 mice were transferred into un-primed Balb/c mice and 24 h later i.p. vaccinated with antigen pulsed DCs. Clonal expansion of antigen specific DO11.10 T cells *in vivo* was assessed based on the proportion of $CD4^+$ and KJ1-26^+ T cells residing in the spleen. After PBS treatment of DCs, the number of DO11.10 T cells represented only 0.04% of the total spleenocyte population. Similarly, vaccination of the recipient mice with untreated or soluble OVA treated DCs with or without MPLA did not increase the detectable number of OVA

specific T cells in the spleen (Fig. 4-12). However, vaccination with DCs pulsed with OVA containing PLGA nanoparticles clonally expanded the donor T cells by 6-9 fold when compared to the PBS group. The expanded KJ1-26$^+$ T cells exhibited a distinct activated and memory phenotype within 3 days of stimulation as they were CD11a^{hi} CD44^{hi} and CD62L^{lo} (Fig. 4-13). A 12-14 fold increase in the detectable number of OVA specific T cells in the spleen could be observed following co-delivery of MPLA along with OVA in PLGA nanoparticles to DCs (Fig. 4-12). The proliferating KJ1-26$^+$ T cells also had a distinct activated phenotype i.e. CD62L^{lo}CD11a^{hi}CD44^{hi} whose expression was higher than observed with DC-OVA nanoparticle vaccination (Fig. 4-13). Also, most of the expanded KJ1-26$^+$ T cells had a higher forward scatter, an indicative of blastogenesis which further suggested that the increase in the number of antigen specific T cells in the spleen was due to clonal expansion and not mere immigration of KJ1-26$^+$ T cells from the peripheral tissues. The control groups immunized with DCs pulsed with MPLA and empty PLGA nanoparticles did not induce any detectable activation of antigen specific T cells in the adoptively transferred Balb/c mice.

4.3.8 DCs treated with PLGA nanoparticles containing BLP25 and MPLA prime naïve T cells and overcome self tolerance mechanisms

The generation of powerful Th1 type primary T cell immune responses *in vitro* and *in vivo* following delivery of model antigen OVA along with MPLA in PLGA nanoparticles to DCs, confirmed the superior applicability of this delivery system for delivering the load of the antigen into DCs for induction of efficient T cell responses. Based on these encouraging results, T cell immune response studies were extended to a more clinically

relevant antigen- BLP25 (which is currently in phase III clinical trials). T cells derived from wild-type and human MUC1.Tg mice (which are naturally tolerant to MUC1) were used to assess whether DCs could process and present the MUC1 antigen from PLGA nanoparticles in such a manner that can overcome self-tolerance mechanisms and elicit T cell proliferative responses. As illustrated in Fig. 4-14A, a strong proliferation of T cells derived from normal wild-type mice could be observed upon treatment of DCs with BLP25-MPLA nanoparticles. DCs treated with these nanoparticles also induced the proliferation of T cells derived from MUC1.Tg mice (Fig. 4-14B). The proliferation of T cells following co-culture with DCs that were either untreated or exposed to soluble BLP25, soluble MPLA, or BLP25 nanoparticles was relatively negligible. DCs treated with empty nanoparticles too could not activate T cells and the physical presence of MPLA was required along with BLP25 in the PLGA nanoparticle formulation for induction of T proliferative responses. MPLA by itself in PLGA nanoparticles stimulated naïve T cells to some extent probably due to its non-specific mitogenic activity (8). These results suggested that delivery of self antigens like MUC1 when delivered alone in PLGA nanoparticles to DCs do not break the self tolerance mechanism. However, incorporation of TLR ligands like MPLA along with the antigen in the PLGA nanoparticle delivery system sufficiently improves the immunogenicity of the vaccine formulation by powerfully activating DCs, which can then overcome self tolerance mechanisms and efficiently prime low affinity MUC1 reactive T cells.

4.4 Discussion

The DC maturation step is a key process for generating immunocompetent DCs and the list of materials that can trigger the maturation stimuli is growing. Efficient DC maturation in addition to inducing effective T cell priming also helps in cross-presentation (11, 12). Thus vaccine antigens that lack immunostimulatory motifs fail to adequately mature DCs which can lead to development of Th2 responses due to the impaired production of IL-12 by DCs (13). Co-delivery of antigens along with immunomodulators like microbial products to DCs is an attractive concept to selectively manipulate the DC microenvironment and favor a desired outcome. The current study employed MPLA, a non-toxic analog of Lipid A and a TLR 4 ligand as the source for the maturation stimuli and immunomodulator. MPLA induces the maturation of DCs and triggers the production of IL-12 (14) which in turn can support the development of Th1 immune responses (15). The current data demonstrated that phenotypic maturation of DCs was adequately achieved with the nanoparticle and soluble mode of MPLA delivery. Consistent with reports of maturation with human cord blood derived DCs (16), a transient upregulation of surface maturation molecules following treatment of DCs with empty PLGA nanoparticles was observed in the current studies. At present it is difficult to explain why in some reports empty PLGA nanoparticles do not induce maturation of DCs while other studies (17) including the current data demonstrated an intermediate maturation. As the nanoparticles had undetectable level of endotoxin it can be hypothesized that the phagocytic process by itself triggers a maturation phenomena. The mature phenotype of DCs was also retained for up to 7 days following MPLA nanoparticle treatment which signified the relative efficiency of this delivery system in

powerfully activating TLRs probably by providing constant TLR4 stimulation through sustained release of MPLA inside the DCs. Enhancement in the expression of maturation molecules on DC surface by itself is insufficient in generating a productive T cell response. Cytokine secretion in the microenvironment of the DC-T cell interaction plays a key role in dictating the nature and outcome of the immune responses generated. The results of this study clearly indicated that despite the induction of phenotypic maturation, soluble MPLA treatment of DCs did not result in the secretion of either of the Th1 or Th2 promoting cytokines. In sharp contrast, DCs targeted with MPLA encapsulated in PLGA nanoparticles secreted IL-12 and also its bioactive form i.e. IL-12p70 that confirmed the effectiveness of this delivery system for supporting polarized Th1 type immune responses. In addition, TNF-α which plays a key role in supporting Th1 responses and induction of cytotoxic T lymphocytes (CTLs) and IL-6, a pro-inflammatory cytokine implicated in suppressing T regulatory cells (T reg) (18) were secreted by DCs following MPLA nanoparticle stimulation. Similarly, visual analysis of the cytokine membrane array data indicated the presence however at a lower level of IL-1α, a pro-inflammatory cytokine and key stimulator of cellular proliferation and IL-13, a T cell survival cytokine each of which are equally important in the initiation and maintenance of immune responses. Chemokines like MIP1α and RANTES that help in the recruitment of DCs, monocytes, and T cells thereby enhancing the magnitude of the generated immune responses were superiorly secreted by MPLA nanoparticle treated DCs. Similarly interferon inducible protein-10 (IP-10), a chemokine that strongly promotes Th1 polarization and retains the recruited T cells in the secondary lymphoid organs and controls effector T cell generation and trafficking (19) was secreted by MPLA

nanoparticle treated DCs. Soluble MPLA treatment of human monocyte derived DCs at doses as high as 100 μg/mL has been reported to induce secretion of IL-12p40 (8). However, IL-12p40 is not the bioactive form of IL-12 and can potentially inhibit the development of Th1 responses (20). The current results clearly indicated an enhanced level of secretion of IL-12p70 by DCs when MPLA was used at 100 ng/mL when delivered in PLGA nanoparticles. In spite of the difference in the experimental models and the source of MPLA, these results indicated the superior applicability of PLGA nanoparticles for delivering the load of immunomodulator to DCs for inducing an effective maturation process. The secretion of IL-12p70 by DCs is sustained and short-lived (21) and if exhausted during T cell encounter polarizes the proliferating T cells towards a Th2 response. This possible *in vivo* scenario was partially addressed by the *in vitro* cytokine release studies that showed the sustained secretion of IL-12p70 and also IL-6 and TNF-α by DCs for up to 48 h following MPLA nanoparticle treatment. The presence of IL-2 and IFN-γ though at very low levels in the supernatant of DC cultures treated with MPLA nanoparticles was particularly interesting as secretion of these two cytokines after pathogen encounter aids in the initiation and maintenance of adaptive immune responses (22). Furthermore, IFN-γ secretion by DCs has also been implicated in the upregulation of IL-12Rβ2 on Th1 cells that can help in a stable Th1 commitment (23) and also in the induction of IFN-γ producing effector $CD4^+$ T cells (24). It is noteworthy to mention that though a transient phenotypic maturation following treatment of DCs with plain PLGA nanoparticles was observed, the functionality of the DCs as assessed by pro-inflammatory cytokine and chemokine secretion remained unaffected.

Effective T cell priming by DCs induces their antigen specific clonal expansion and differentiation into cytokine secreting effector and memory T cells. In this context particle mode of antigen delivery to DCs was found to be more efficient than the soluble antigen for uptake and subsequent antigen presentation (25). In the current study, the ability of DCs loaded with PLGA nanoparticles containing antigen and MPLA to stimulate primary T cells was assessed. Antigen delivery to DCs in PLGA nanoparticles induced primary T cell proliferative responses that were 500-1000 fold higher than observed following soluble antigen treatment which indicated that PLGA nanoparticles offer a superior mode of antigen transfer to DCs. These observations also implied that the protein antigen encapsulated in PLGA nanoparticles was adequately released inside the DC following phagocytosis and was processed and efficiently presented in context of MHC class II as peptide epitopes to antigen specific $CD4^+$ T cells. The secretion of IL-2 and IFN-γ by the proliferating $CD4^+$ T cells indicated the promising role the nanoparticle mode of antigen and MPLA delivery holds for generating cytokine producing effector T helper cells that aid in the generation of CTL responses and initiate $CD8^+$ T cell memory (26, 27). The potent priming of naive T cells by DCs when MPLA was co-delivered along with OVA in the nanoparticle formulation may at least be in part due to a direct effect of superior co-stimulation initiated by pro-inflammatory cytokine secretion (TNF-α and IL-1α) that enhances the expression of CD40 on DCs (28). CD40 mediated signaling can furthermore enhance the expression of CD80 and CD86 co-stimulatory molecules on DC surface thus increasing the extent of co-stimulation available to T cells. Likewise, a 100 fold enhancement in the signaling process initiated by the MHC and TCR complexes was detected following engagement of CD28 on T cells.

The *in vitro* potential of DCs loaded with PLGA nanoparticles containing antigen and adjuvant to generate primary T cell responses has been tested and described but the *in vivo* applicability of the nanoparticle loaded DCs to elicit immune responses was yet to be confirmed. This crucial issue was addressed using the DO11.10 T cell adoptive transfer model. As illustrated in Fig. 4-12, the clonal expansion of antigen specific T cells following vaccination with DCs pulsed with antigen in PLGA nanoparticles was clearly observed while the incorporation of MPLA along with the antigen induced a modest increase in stimulation. The difference in clonal expansion of antigen specific T cells following vaccination with DCs pulsed either with OVA nanoparticles or OVA-MPLA nanoparticles was however not significant ($P > 0.05$). This modest increase in antigen specific T cell number in the spleen following incorporation of MPLA along with OVA is still appreciable as the primed clonotype specific T cells detected in the spleen represents a very minute percentage of the whole spleen T cell population. Furthermore, it is very much possible for the activated T cells to have migrated to peripheral tissues reducing the number of $CD4^+/KJ1\text{-}26^+$ T cells residing in the spleen. A similar pattern of enhanced T cell activation in the presence of MPLA in the antigen containing nanoparticle formulation has been reported (29) which indicated that MPLA powerfully activated APCs which lead to enhanced and prolonged priming of T cells. Generation of CTL responses was also recently reported in mice that were vaccinated with DCs loaded with antigen encapsulated in PLGA microparticles (30).

Our ultimate goal is to efficiently target DCs generated *ex vivo* with cancer antigens and adjuvants through their delivery in PLGA nanoparticles for application in cancer immunotherapy through DC adoptive therapy. As cancer antigens are often self antigens,

the mode of antigen delivery to DCs should be powerful enough to overcome self tolerance mechanisms. BLP25 was chosen as the tumour antigen due to its significance as a cancer vaccine candidate in the immunotherapy of $MUC1^+$ tumours (31). The current results indicated that DCs targeted with BLP25 in solution form are inefficient in priming MUC1 specific T cells. However, delivery of BLP25 in PLGA nanoparticles to DCs overcomes the self tolerance mechanisms and induces the proliferation of T cells derived from MUC1 transgenic mice *in vitro*. This process however required TLR signaling through co-delivery of MPLA in the PLGA nanoparticle formulation.

In conclusion, the current results demonstrated that TLR mediated stimulation of DCs through delivery of MPLA in PLGA nanoparticles induced the phenotypic and functional maturation of DCs. Furthermore, delivery of antigen and MPLA encapsulated in PLGA nanoparticles to DCs superiorly generated Th1 polarized primary T cell immune responses *in vitro* and *in vivo*. Finally, this mode of antigen delivery to DCs can potentially overcome self tolerance mechanisms. Therefore, delivering tumour antigens along with TLR ligands like MPLA in PLGA nanoparticles to DCs can be considered as a highly effective strategy for the development of tailored cellular vaccines for application in cancer immunotherapy.

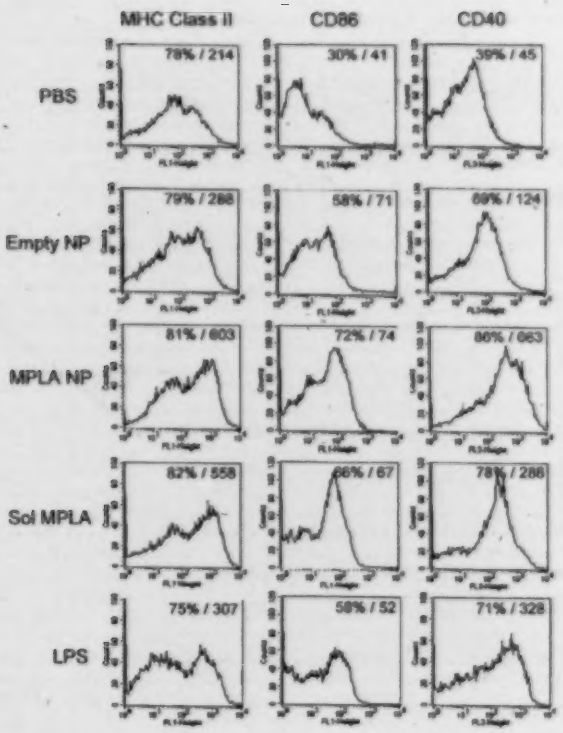

Figure 4-1. Expression of MHC class II, CD86, and CD40 molecules on DCs upon treatment with MPLA either in free form or via PLGA nanoparticle. Day 7 DCs were treated with various formulations for 24 h and the expression of various markers determined by FACS. The numbers within the histogram plot represent the percent positive cells and the mean fluorescent intensity for the marker respectively. Gating was performed on the live cell population. One representative experiment out of three is shown

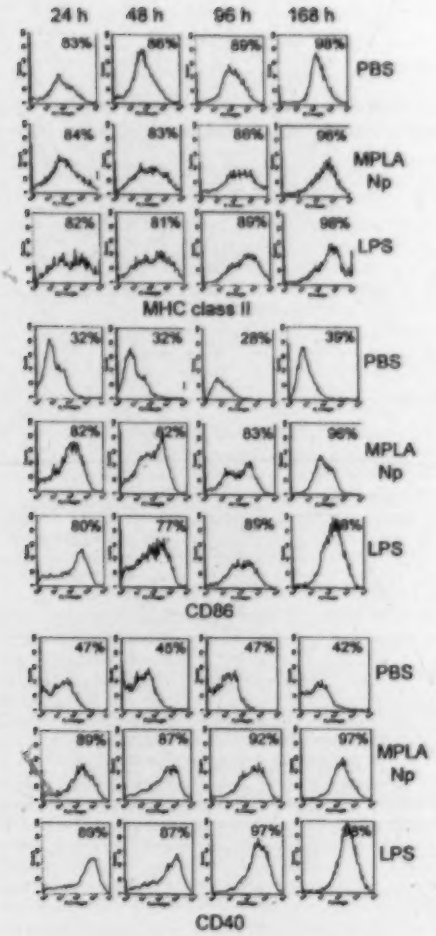

Figure 4-2. Kinetics of expression of maturation markers on DCs. Day 7 DCs were incubated with PBS, MPLA nanoparticles, or LPS for 24 h. Cells were then harvested and propagated for up to the indicated time points and the expression of the markers analyzed by FACS. The numbers within the histogram plot represent the percent positive cells. Gating was done on the live cell population. One representative experiment out of two is shown.

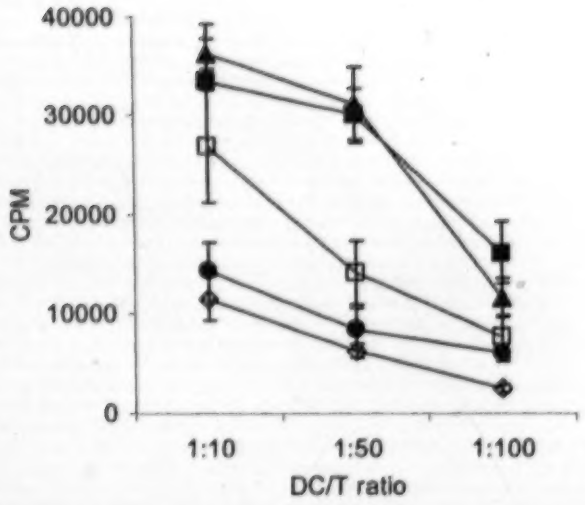

Figure 4-3. Allostimulatory capacity of DCs treated with MPLA formulations. Day 7 DCs ($CD11c^+$) were left untreated (◊) or treated with 50 μg/ml of PLGA nanoparticles without (●) or with MPLA (■), or soluble MPLA (□), or LPS (▲). The cells were harvested after 24 h, irradiated and incubated with 2×10^5 allogeneic T cells. The T cell number was fixed and the DC number varied to get the desired DC:T cell ratio. The proliferation of T cells was measured by ^{3}H-thymidine incorporation. One representative experiment out of two is shown.

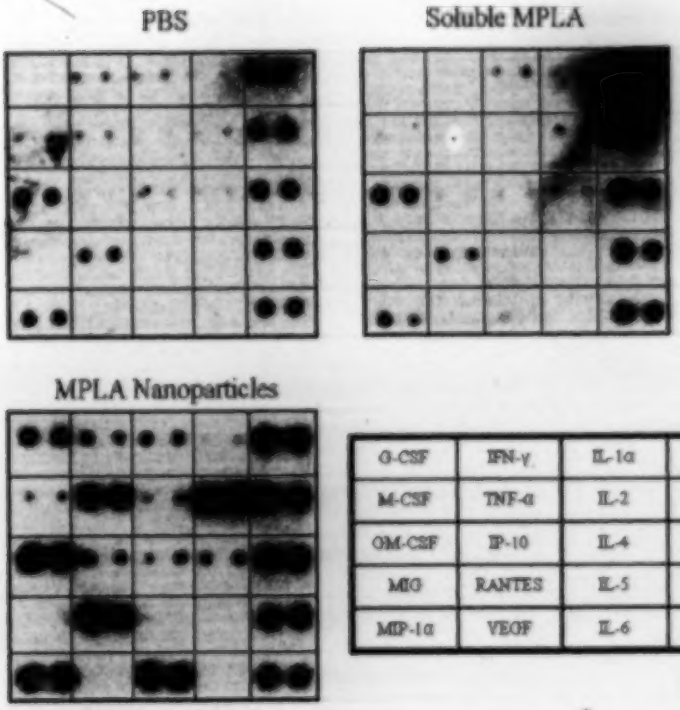

Figure 4-4. Secretion of cytokines and chemokines by DCs. Day 7 DCs ($CD11c^+$) were incubated with PBS or soluble MPLA or MPLA nanoparticles (dose of MPLA- 100 ng/mL) for 24 h. The supernatants were then tested for the presence of cytokines and chemokines by membrane arrays. Results are representative of two independent experiments

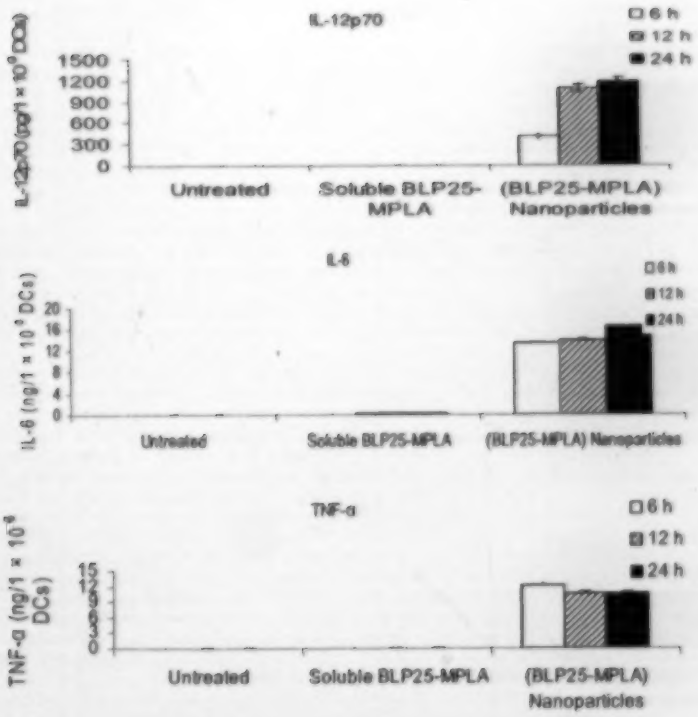

Figure 4-5. Kinetics of secretion of cytokines by DCs. Day 7 DCs ($CD11c^+$) were incubated with PBS or soluble MPLA or MPLA nanoparticles for up to the designated time points. The supernatants were then tested for the presence of cytokines by quantitative ELISA kits. Error bars represent S.D of the mean of wells in triplicates. One representative experiment out of two is shown.

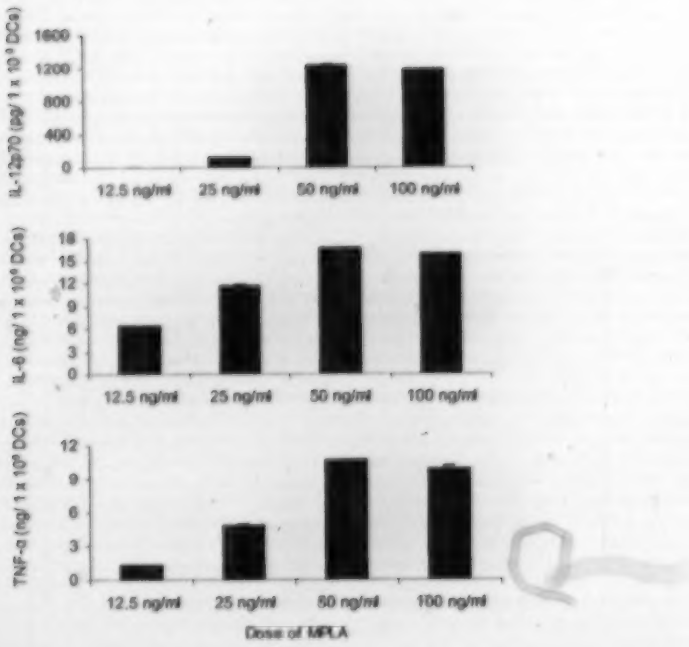

Figure 4-6. Secretion of cytokines by DCs following incubation with PLGA nanoparticles containing different doses of MPLA. Day 7 DCs ($CD11c^+$) were incubated with PLGA nanoparticles containing varying doses of MPLA for 24 h. The supernatants were tested for the presence of cytokines by quantitative ELISA kits. Error bars represent S.D of the mean of wells in triplicates. One representative experiment out of two is shown.

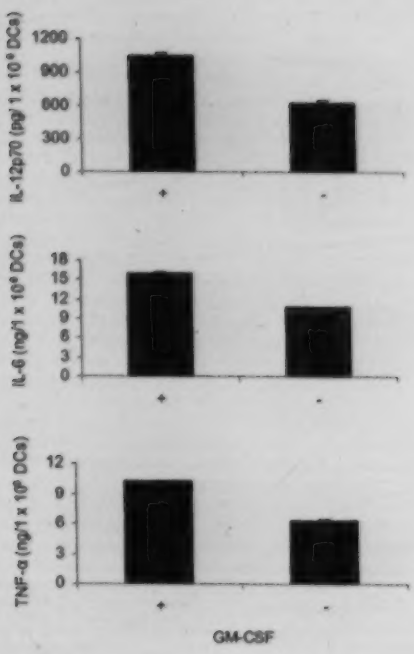

Figure 4-7. Influence of GM-CSF in the culture medium on the secretion of cytokines by DCs. Day 7 DCs ($CD11c^+$) were incubated with MPLA containing PLGA nanoparticles in the presence or absence of GM-CSF for 24 h. Cytokines in the supernatant were analyzed by quantitative ELISA kits. Error bars represent S.D of the mean of wells in triplicates. One representative experiment out of two is shown.

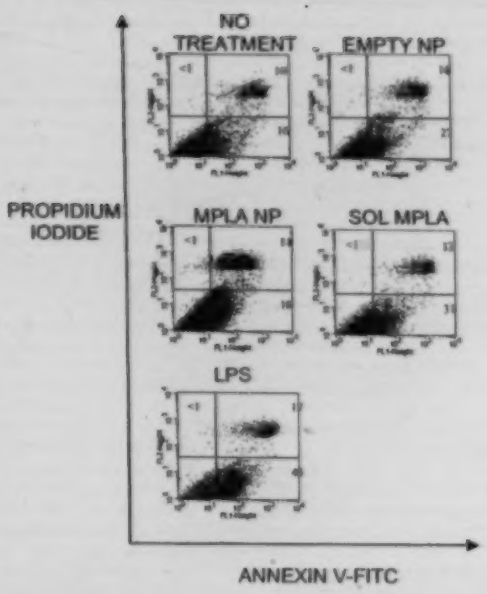

Figure 4-8. Viability of DCs treated with PLGA nanoparticles. Day 7 DCs were incubated with PBS or PLGA nanoparticles with or without MPLA or soluble MPLA or LPS for 24 h. The cells were harvested and incubated with AnnexinV-FITC and PI and analyzed by FACS. Gating was done on the total cell population. One representative experiment out of three is shown.

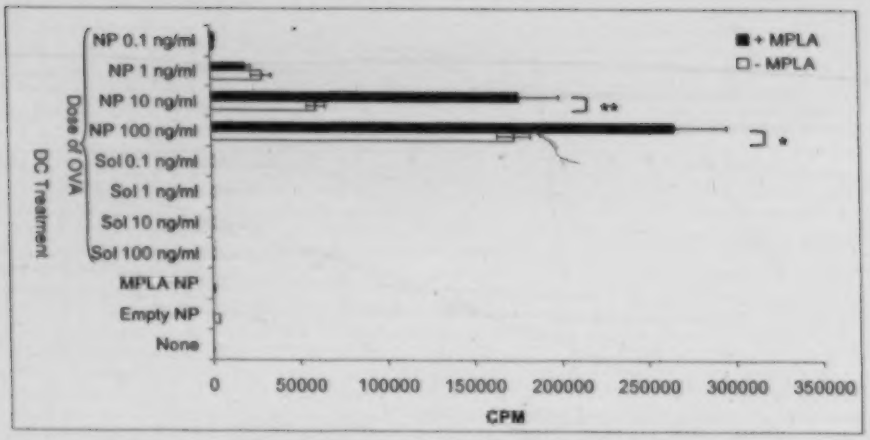

Figure 4-9. Primary T cell responses against OVA following *in vitro* immunization with DCs. DCs were incubated with OVA contained in PLGA nanoparticles or in solution. Some OVA nanoparticles were formulated with MPLA. After 24 h of incubation, DCs were harvested and cultured with DO11.10 $CD4^+$ T cells for 72 h and the proliferation measured by ^{3}H-thymidine incorporation. Statistical difference in T cell proliferation between groups treated with OVA in presence or absence of MPLA in the NP formulation. *$P<0.01$, **$P<0.005$. Error bars represent S.D of the mean of triplicate wells. One representative experiment out of two is shown.

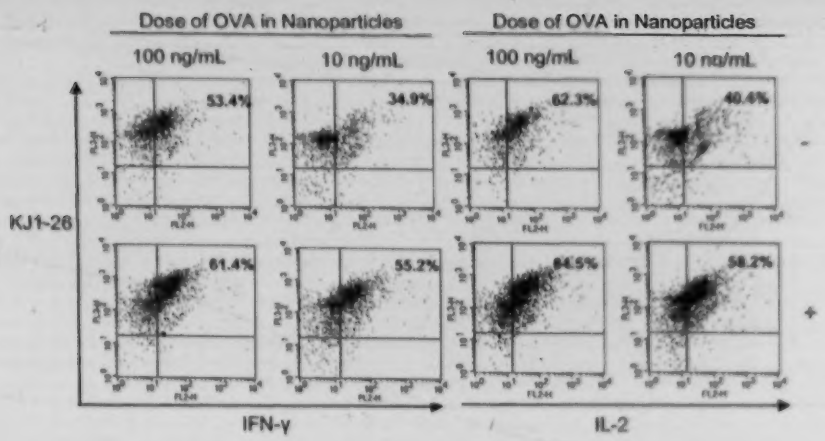

Figure 4-10. Intracellular secretion of IL-2 and IFN-γ by T cells following *in vitro* immunization with DCs. DCs were incubated with OVA contained in PLGA nanoparticles along with or without co-encapsulated MPLA. After 24 h, the cells were harvested and co-cultured with DO11.10 $CD4^+$ T cells and the secretion of cytokines detected by intracellular cytokine staining. The background secretion of both of these cytokines by T cells following incubation with unpulsed DCs was below 10%. One representative experiment out of two is shown.

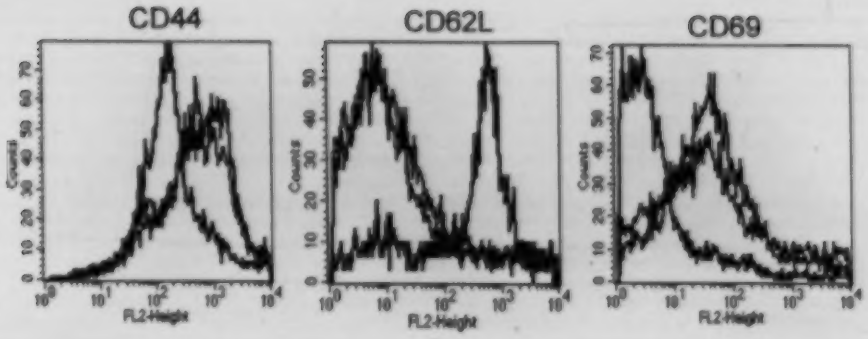

Figure 4-11. Expression of activation and memory markers on T cells following stimulation by DCs. The expression of markers on T cells following stimulation with DCs that were incubated with PBS (black) or [OVA] NP (green) or [OVA+MPLA] NP (red) is shown. One representative experiment out of two is shown.

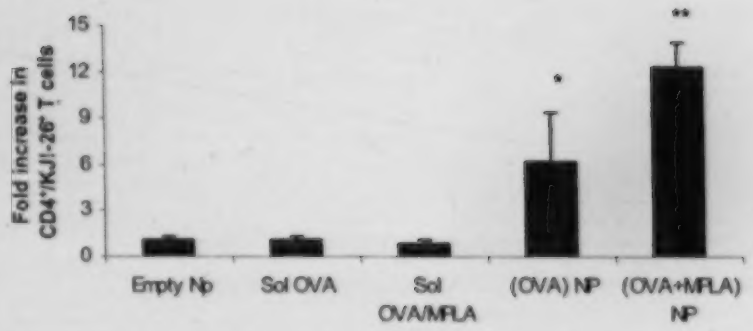

Figure 4-12. Clonal expansion of antigen specific T cells *in vivo*. DCs were treated with various formulations for 12 h and vaccinated i.p into mice that were adoptively transferred with $CD4^+$ T cells obtained from DO11.10 mice. The proportion of $CD4^+/KJ1\text{-}26^+$ T cells in the spleen was analyzed after 3 days by FACS and represented as fold increase of antigen specific T cells as compared to PBS treatment. The values are mean of three independent experiments ± SD. Statistical difference in T cell clonal expansion between (OVA) NP or (OVA+MPLA) NP treatment group and 'Empty NP treatment'. $*P<0.05$, $**P<0.01$. Error bars represent S.D of the fold increase in antigen specific T cells. One representative experiment out of three is shown.

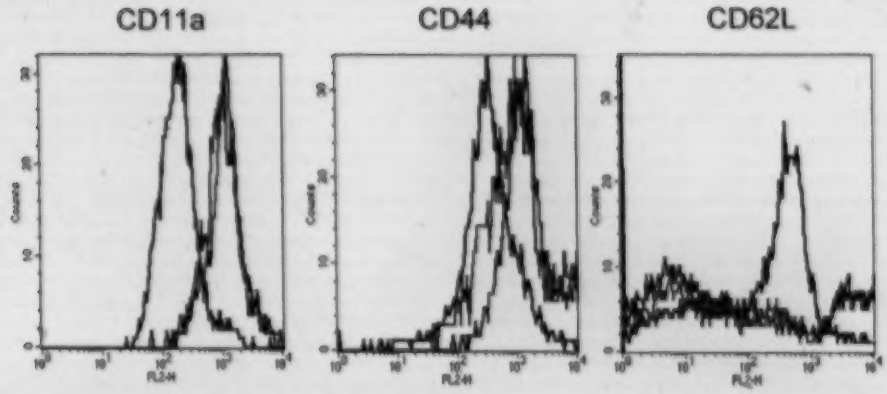

Figure 4-13. Expression of activation and memory markers on T cells following DC vaccination. The expression of the markers on $CD90.2^{+}KJ1\text{-}26^{+}$ gated live T cells obtained from the spleens of mice vaccinated with DCs is shown. DCs were incubated with either PBS (black) or [OVA] NP (green) or [OVA+MPLA] NP (red) prior to vaccination. One representative experiment out of three is shown.

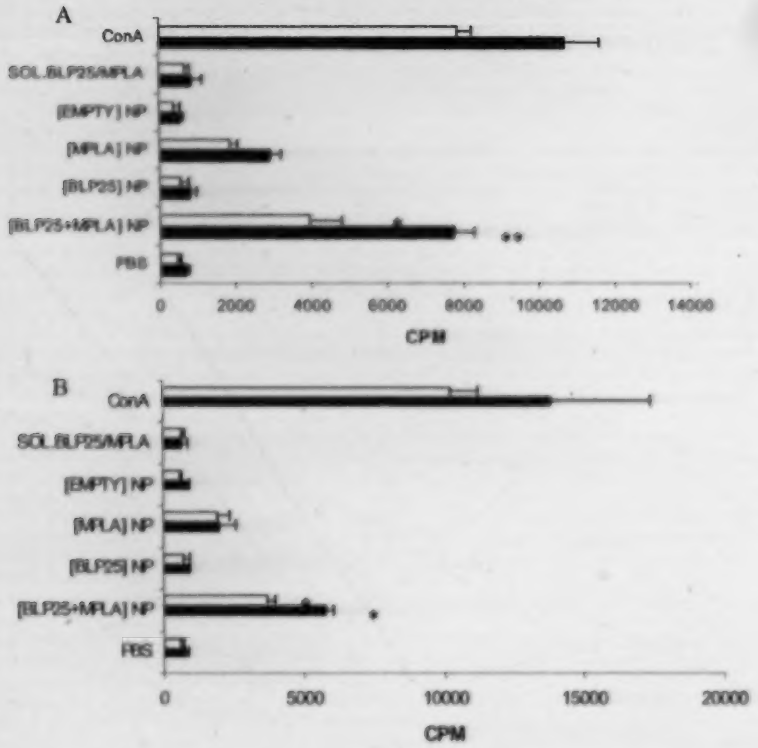

Figure 4-14. Primary T cell responses against MUC1 following *in vitro* immunization with DCs. DCs were incubated with BLP25 contained in PLGA nanoparticles or in solution with or without MPLA. Some DC groups were treated with PBS (APC group) or empty nanoparticles or MPLA nanoparticles. After 24 h of incubation, DCs were co-cultured with T cells obtained from either normal mice (A) or human MUC1. Tg mice (B). Con A treatment was used as positive control. T cell proliferation was measured by ^{3}H-thymidine incorporation. Black and white bars represent DC to T cell ratios of 1:5 and 1:10 respectively with the T cell number kept constant. Statistical difference in T cell proliferation between DC groups treated with [MPLA] NP and [BLP25+MPLA]. $^{*}P<0.05$, $^{**}P<0.01$. Error bars represent S.D of the mean of triplicate wells. One representative experiment out of three is shown.

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Chapter Five

General Discussion, Conclusions, and Future Directions

5.1 Discussion

In the past decade, a major emphasis in vaccine research was elucidating immunological mechanisms underlying host defense against pathogens. Recent advances in immunology have clearly shown that the nature of an ensuing immune response is largely influenced by the manner in which the immune system encounters the antigen [1]. Thus, it may be possible to generate radically opposing effects such as elimination or tolerance against a pathogen or tumour cell by controlling the microenvironment of antigen encounter. Therefore, targeting antigens to the appropriate immune cells especially DCs and controlling the microenvironment of antigen capture and presentation are important issues in vaccine development [2, 3]. An efficient vaccine not only requires the right antigen but also an effective delivery system. Pharmaceutical vaccine delivery systems that can create the desired environment in which the antigen is seen by DCs would advance the design of successful vaccines against a number of diseases.

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T cell mediated immune responses play a pivotal role in mediating cancer rejection [4]. Therefore, development of a strategy that can efficiently generate anticancer T cell immune responses was chosen as the major focus of this research project. In this context, a MUC1 based poly(D,L-lactic-co-glycolic acid) (PLGA) nanoparticle vaccine formulation designed for eliciting robust anti MUC1 T cell responses was investigated. The current work demonstrated that PLGA nanoparticles are efficient antigen delivery vehicles for targeting therapeutically relevant antigens like MUC1 to the immune system (Chapter 2). The likely reason for the efficiency of this delivery system in eliciting potent anti MUC1 immune responses *in vivo* might be that, the antigen is being superiorly delivered to DCs *in vivo* when encapsulated in PLGA nanoparticles. Previous work done

by our group demonstrated the delivery of PLGA nanoparticles to DCs when administered *in vivo* [5]. Furthermore, the particulate nature of the antigen ensures that a high antigen dose is maintained within DCs after its uptake. The results in Chapter 3 support this hypothesis, as a thousand fold higher antigen transfer into DCs could be achieved when the antigen was encapsulated in PLGA nanoparticles than in free or solution form. Likewise, the enhanced transfer of antigen into DCs might be responsible for their ability to generate potent T cell immune responses *in vitro* and *in vivo* (Chapter 4).

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Most of the cancer associated antigens are poorly immunogenic self molecules. Tolerance against self antigens is an important barrier for anticancer T cell immunity. Toll like receptor (TLR) ligands are known to overcome self tolerance mechanisms through suppression of regulatory T (T reg) cells [6]. Therefore, a critical concept in cancer vaccine development is to deliver the TLR ligand and the antigen to the same DC. This strategy would enable the DC that has internalized the antigen and received the maturation stimulus to efficiently interact with self reactive T cells. The observations in chapter 2 clearly signified the importance of co-delivery of TLR ligands for overcoming tolerance against MUC1. The proliferation of anti MUC1 T cells must have resulted from enhanced maturation of DCs after phagocytosis of nanoparticles containing MUC1 antigen and monophosphoryl lipid A (MPLA). The potent effects of MPLA containing nanoparticles on DC maturation are clearly demonstrated in chapter 4. These studies indicated that phenotypic and functionally mature DCs that secreted interleukin- 12 (IL-12) and other key Th1 promoting cytokines and chemokines were efficiently generated following treatment with MPLA containing PLGA nanoparticles. *In vitro* DC

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immunization data with MUC1 in chapter 4 further signified the co-delivery requirement of TLR ligands in overcoming tolerance as no anti MUC1 T cell proliferation could be detected in the absence of MPLA in the nanoparticles.

The presence of TLR ligands is also critical for the generation of robust T helper 1 (Th1) immune responses. The results in chapter 2 demonstrated this concept as immunization of mice with nanoparticles containing MUC1 and MPLA generated antigen specific T cells that secreted high amounts of Interferon-γ (IFN-γ, indicative of Th1 type immune responses). Furthermore, the same effect was observed in the *in vitro* experiments in chapter 4, where delivery of MPLA along with ovalbumin (OVA) antigen in nanoparticles to DCs generated higher numbers of OVA specific T cells secreting IL-2 and IFN-γ. Likewise, the presence of MPLA along with the antigen enhanced the T cell stimulatory potential of DCs by almost two fold in the *in vitro* and *in vivo* DC vaccination experiments (chapter 4). Furthermore, in addition to adjuvancy mediated through MPLA, the PLGA nanoparticle delivery system was also well documented to aid in promoting Th1 responses [7, 8]. Hence, the observed Th1 responses in our studies might due to the synergistic effects of TLR signaling by MPLA and enhanced delivery by PLGA nanoparticles.

In addition to Th responses, cytotoxic T lymphocyte (CTL) responses play a critical role in mediating cell-mediated immunity and are crucial for combating cancer. The generation of CTL responses by exogenous particulate antigens requires cross-presentation of the antigen for major histocompatibility complex (MHC) class I presentation. The phagosome to cytosol pathway of antigen trafficking has been implicated in cross presentation of antigens located in the phagosome (discussed in

chapter 1). The study in chapter 3 which tracked the cellular location of a fluorescent probe encapsulated in PLGA nanoparticles demonstrated that intracellular localization of nanoparticles was achieved within DCs after uptake. Based on the focal planes, the internalized nanoparticles were most likely located in the phagosomes. These results are complimentary to previous studies that demonstrated that PLGA particles are localized in the phagosomes after uptake by macrophages and cytoplasmic delivery of the encapsulated probes could be achieved. Therefore this study demonstrated that probes encapsulated in PLGA nanoparticles are capable of cytoplasmic delivery within DCs, which can be beneficial for loading onto MHC class I compartments through cross-presentation.

The important goal of the research project was to generate *ex vivo* antigen and adjuvant loaded DCs and test its potential for DC adoptive therapy. The results in chapter 4 indicated that, *ex vivo* antigen loaded DCs were capable of robust priming of antigen specific T cells *in vivo*. Similar to the enhanced T cell proliferative responses that resulted due to MPLA in nanoparticles, robust expansion of T cells *in vivo* could be detected by this delivery approach. These observations thus demonstrated the potential of nanoparticulate antigen loaded DCs for *in vivo* application in cancer immunotherapy. Direct measurement of $CD8^+$ T cell responses and ability of this strategy in regression of established tumours was not performed in this study. However, the current observations have proved the applicability of this 'adoptive therapy' approach in supporting the development of Th1 responses. A recent study indicated that CTL responses could be efficiently elicited by this mode of antigen delivery to DCs *in vivo* [9].

5.2 Conclusions

1. Single emulsion PLGA nanoparticles entrapping a 25 mer human MUC1 lipopeptide (BLP25) and MPLA have been prepared by the solvent evaporation method. The nanoparticle formulation induced potent Th1 type immune responses *in vivo* and had overcome self tolerance mechanisms, indicating it as a promising anticancer vaccine formulation.
2. PLGA nanoparticles are efficiently taken up by DCs *in vitro* through phagocytosis and are localized in their intracellular compartments.
3. Phenotypic and functionally mature DCs that produce Th1 promoting cytokines and chemokines can be effectively generated through delivery of MPLA in PLGA nanoparticles.
4. DCs targeted *in vitro* with antigen and MPLA co-encapsulated in PLGA nanoparticles clonally expand antigen specific T cells *in vitro* and *in vivo* and transform naïve T cells into Th1 cytokine secreting effector T cells.
5. DCs loaded *in vitro* with PLGA nanoparticles encapsulating BLP25 and MPLA are capable of overcoming the self tolerance mechanisms against MUC1.

5.3 Future Avenues of Investigation

PLGA copolymer has been extensively investigated for delivering a variety of drugs. The current studies demonstrate that this biodegradable polymer also holds excellent potential for developing novel vaccine technologies, especially for therapeutic vaccines for cancer. However, success of this approach will require careful design of the delivery strategy in order to recruit the full spectrum of cell-mediated immune responses and

overcome cancer immune evasion. With further study and exploration of this delivery system, the potential of PLGA nanoparticles in the development of vaccines against various diseases that could benefit from cellular immune responses are enormous. Some of the key areas that could be benefited are discussed below.

The current observations indicated that PLGA nanoparticles encapsulating MUC1 lipopeptide and MPLA can overcome self tolerance mechanisms and activate MUC1 specific $CD4^+$ T cell responses strongly, but provide weak $CD8^+$ T cell responses. Although $CD8^+$ T cells are the primary mediators of antitumour immunity, the importance of $CD4^+$ T cells in controlling antitumour immune responses including CTL responses is beginning to be understood [10]. However, weak $CD8^+$ T cell responses, is still a limiting factor for the success of the vaccine formulation. One potential reason for the weak $CD8^+$ T cell responses is that the tandem repeat domain of MUC1 does not contain strong anchor motifs for MHC class I binding. However, the cytoplasmic domain and the signal peptide sequences contain better epitopes for binding within the MHC class I groove [11, 12]. The signal peptide contains MHC class I epitopes, and $CD8^+$ T cells against this sequence have been detected [13]. The cytoplasmic domain peptide has several epitopes potentially presented by MHC class I molecules [12]. Thus, MUC1 based vaccine formulations should look at encapsulating the cytoplasmic domain and signal peptide sequences of MUC1 along with the MUC1 tandem repeat (all as lipopeptides) along with MPLA in PLGA nanoparticles and assess the immunogenicity of the formulation for inducing $CD4^+$ and $CD8^+$ T cell responses against MUC1.

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One of the key factors limiting the success of anti cancer vaccines is the presence of an immunosuppressive milieu that prevents generation of therapeutic immune responses.

Tumour antigen specific T reg cells are recruited by the tumour, which inhibit the effector functions of anticancer T cells by maintaining peripheral T cell tolerance against cancer antigens. Current cancer immunotherapy strategies are looking at identifying novel ways to inhibit T reg activity. Recent studies indicated cyclophosphamide as a potent inhibitor of T reg activity. Cyclophosphamide treatment can help recruit high avidity tumour antigen specific T cells to the tumour site and keep the T reg cells at bay [14]. This effect was partly due to depletion of T reg cells and inhibition of T reg function [15]. Thus, it is a worthwhile strategy to include low dose cyclophosphamide treatment in conjunction with MUC1 cancer vaccines for anticancer immunity. In addition, direct delivery of PLGA nanoparticles containing cyclophosphamide analogues to the tumour site ensues that a sustained release of the drug is maintained. This mode of delivery will have the potential in reducing T reg activity at the tumour site and favor expansion of antigen specific T cells induced by nanoparticle formulations of MUC1.

One more important factor that promotes tumour survival and can be a key target of anticancer vaccines is signal transducers and activators of transcription (STATs). STATs are cytoplasmic signaling molecules that upon phosphorylation by upstream kinases, dimerize and translocate to the nucleus where they function as transcription factors. In a variety of human cancers, STAT3 is constitutively activated [16, 17] and promotes tumour growth, resistance to apoptosis and immune evasion [18-20]. STAT3 activation in DCs also impairs the production of IL-12 and thereby suppresses the development of Th1 responses. There has been considerable evidence where tumour growth could be inhibited by inhibiting STAT3 activity [22]. Thus, strategies that can inhibit STAT3 activity will be beneficial in generation of functionally potent DCs. Novel STAT3 inhibitors like

small interfering RNA (siRNA) [22] and just another kinase 2 (JAK2)/STAT3 inhibitor like cucurbitacin 1 [23] can be encapsulated along with the MUC1 lipopeptide antigen and MPLA in PLGA nanoparticles. This nanoparticle vaccine formulation has the potential to restore activity in tolerogenic DCs and enhance DC maturation, both of which are essential in overcoming self tolerance.

The robust expansion of T cells by nanoparticulate antigen loaded DCs opens up new possibilities in the area of adoptive T cell therapy [24, 25]. The number of tumour antigen specific T cells *in vivo* is extremely low and the expansion of these T cells is inhibited by immunosuppressive environment created by tumour. Therefore, robust expansion of these rare T cells *ex vivo* by DCs loaded with nanoparticulate antigens and adjuvants and infusing the expanded effector T cell populations to the patient is an attractive concept for cancer immunotherapy.

In addition to implications for the immunotherapy of cancer, the nanoparticulate antigen delivery strategy to DCs has a more general application. Defective DCs are present during viral infections of human immunodeficiency virus (HIV) and hepatitis B and C. Propagation of DCs *ex vivo* from progenitor cells can generate functionally potent DCs that can aid in clearing the viral infection. In addition, a strong Th1 response is required to alleviate the viral disease condition. We have previously shown that hepatitis B viral antigen along with MPLA when co-delivered in PLGA nanoparticles generates potent Th1 responses *in vivo*. Therefore, an attractive strategy in viral immunotherapy is to load DCs generated *ex vivo* with PLGA nanoparticles containing viral antigens and adjuvants. These vaccine loaded DCs can then be employed for *in vivo* vaccination to alleviate the disease condition.

The DC-T cell signaling network plays an important role in dictating the nature of the ensuing immune response. Identifying novel pathways involved in this signaling process and exploiting them to our advantage through careful selection of modulators and delivery parameters is critical for developing smart vaccines. Future prospects of DCs loaded with PLGA nanoparticle vaccines remain numerous, including treatment of HIV and overcoming tolerance in autoimmunity and transplantation. The current understanding of DC biology has uncovered many facets of its immune regulatory activities. With continued progress in this field, it is expected that PLGA nanoparticle loaded DCs will play a vital role in the development and design of future vaccines.

5.4 References

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